



## THESIS / THÈSE

### MASTER IN BIOCHEMISTRY AND MOLECULAR AND CELL BIOLOGY RESEARCH FOCUS

#### Transcriptional interactions between RNA polymerases II and III

Lebrun, Ysaline

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**Faculté des Sciences**

**Transcriptional interactions between RNA polymerase II and III**

**Mémoire présenté pour l'obtention  
du grade académique de master 120 en biochimie et biologie moléculaire et cellulaire**  
Ysaline LEBRUN  
Janvier 2019

**Université de Namur**  
**FACULTE DES SCIENCES**  
Secrétariat du Département de Biologie  
Rue de Bruxelles 61 - 5000 NAMUR  
Téléphone: + 32(0)81.72.44.18 - Téléfax: + 32(0)81.72.44.20  
E-mail: joelle.jonet@unamur.be - <http://www.unamur.be>

## **Transcriptional interactions between RNA polymerase II and III**

LEBRUN Ysaline

### Résumé

La transcription réalisée par l'ARN polymérase III (Pol III) est un des facteurs clés influençant, entre autres, la synthèse protéique ainsi qu'un grand nombre d'activités cellulaires. La régulation de cette polymérase reste pourtant un domaine peu connu. Dans le but de fournir de nouvelles suggestions de voie de régulation, des expérimentations ont été menées en prenant pour point de départ un mutant de Pol III montrant un net défaut de croissance et une charge réduite en précurseurs d'ARN de transfert, l'un des types de transcrits de Pol III. Dans la mesure où ce mutant provient de l'addition d'un peptide sur l'une des sous-unités de Pol III, Rpc25, la fonction de cette sous-unité a d'abord été étudiée. Sur base de ces résultats et de la structure du complexe Pol III, son rôle a été élucidé et sa mutation a été mise en relation avec un défaut touchant l'initiation de la transcription. Nous avons également montré que cette mutation n'a pas d'impact sur l'activité du complexe TORC1, malgré la régulation présumée de ce complexe par la charge en précurseurs d'ARN de transfert dans la cellule. En revanche, elle induit la sensibilité à la surexpression d'une protéine de stabilisation des précurseurs d'ARN de transfert, Sla1.

En parallèle, des suppresseurs naturels du phénotype de défaut de croissance ont été isolés sur base de cette souche mutante. Ils ont également été séquencés afin de pouvoir localiser précisément les nouvelles mutations qu'ils avaient acquises. Sur base de l'identification des gènes affectés par ces mutations, il est possible de montrer que certains acteurs apparentés à l'ARN polymérase II ont un impact sur la transcription par Pol III. Nous proposons donc l'existence d'une régulation inter-polymérase touchant Pol III.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire

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**Promoteur:** HERMAND Damien

**Université de Namur**  
**FACULTE DES SCIENCES**  
Secrétariat du Département de Biologie  
Rue de Bruxelles 61 - 5000 NAMUR  
Téléphone: + 32(0)81.72.44.18 - Téléfax: + 32(0)81.72.44.20  
E-mail: joelle.jonet@unamur.be - <http://www.unamur.be>

## **Transcriptional interactions between RNA polymerase II and III**

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### Abstract

Transcription by the RNA polymerase III (Pol III) is one of the key determinants affecting protein synthesis regulation, among other things. However, the regulation of this polymerase remains poorly understood. In order to open new leads on Pol III regulation paths, experiments were performed based on a Pol III mutant showing a marked growth defect and reduced amount of one type of Pol III transcripts, transfer RNA precursors. As this mutant particularity arises from addition of a peptide on the Pol III subunit Rpc25, we studied the role of this subunit in transcription. On the basis of our results and of Pol III complex structure, the role of this subunit was elucidated and its mutation was linked to a defect in transcription initiation. We also show that its mutation has no significant impact on the activity of a particular complex, TORC1, despite that this complex is supposed to be regulated by the concentration of transfer RNA precursors. Reversely, mutation of Rpc25 causes a sensibility of this strain to overexpression of a protein stabilizing transfer RNA precursors, Sla1.

In parallel, naturally emerging suppressors of the growth defect phenotype of this mutant were isolated and sequenced in order to precisely map the acquired suppressive mutations. Based on the identified mutations, we show that some Pol II related actors have an impact on Pol III transcription. Thus, we propose the existence of a trans-polymerases regulation impacting Pol III.

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Je remercie tout d'abord le professeur Damien Hermand pour m'avoir accueillie au sein de son laboratoire et m'avoir donné l'occasion d'apprendre au sein d'une équipe si conviviale. Je le remercie également pour ses conseils, ses idées, la qualité de son encadrement et sa capacité à voir quelque chose d'intéressant même dans le résultat le plus brumeux.

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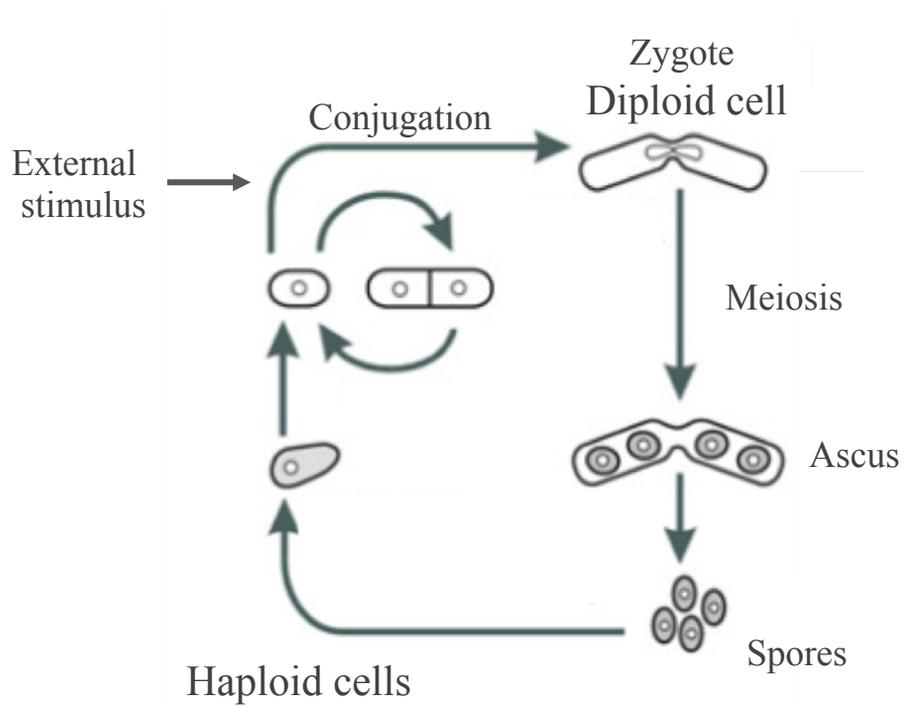
# Objectives

The master thesis general objectives are based on previous unpublished results obtained during the PhD thesis of Carlo Yague-Sanz. This work revealed that the addition of a small peptide (the flag) at the C-terminal end of a subunit of the RNA polymerase III (Pol III), Rpc25, leads to a marked defect in pre-tRNA production and an impaired growth in the resulting strain, *rpc25-flag*. Surprisingly, combining this allele with mutations affecting the phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (Pol II) suppressed these phenotypes, suggesting a connection between Pol III and Pol II, possibly through the CTD.

In order to shed light on this putative new mode of Pol III regulation, the main objective of this master thesis is to identify naturally emerging extragenic suppressors of the *rpc25-flag* mutant growth defect phenotype by whole-genome sequencing. Indeed, identification of mutations that took place in these suppressors is a powerful tool to identify genes whose protein or RNA product negatively affects transcription by Pol III. We also performed experiments in order to shed light on the mechanism by which these suppressors improve growth.

Our secondary objective for this master thesis is to better characterize the *rpc25-flag* mutant. More specifically, we intend to determine the molecular basis of the Pol III defect in this mutant. Therefore, we assessed the abundance of Rpc1 (the catalytic subunit of Pol III) in the *rpc25-flag* mutant background. We also assessed Pol III binding to its target gene in this mutant.

The following introduction aims at giving the necessary background for experimentations that will be discussed later, as well as insight into elements of Pol III regulation, potential or asserted, and more specifically Pol III regulation related to Pol II. Background elements on the Pol II CTD and on other components that regulate Pol II and Pol III, for example the state of the chromatin, will also be provided.



**Figure 1: An overview of *S. pombe* life cycle.** Haploid cells exit their standard mitotic life cycle to perform conjugation, in response to external stimulus. The diploid zygote formed enter meiosis to form four haploid spores contained inside an ascus [2,3] .

# *Introduction*

## **Schizosaccharomyces pombe**

First, an introduction of the model organism that will serve through this study seems to be necessary, as specific characteristics of this organism will impact experimentations. *Schizosaccharomyces pombe*, also referred as “fission yeast”, is a single-celled free-living archiascomycete that is used as a model organism in molecular biology. The term “yeast” refers to a broad range of unicellular fungi that includes two major model organisms: our model, *S. pombe*, and the budding yeast *Saccharomyces cerevisiae* which is also known as the brewer's or the baker's yeast. Although they are both referred to as “yeast”, they are only distantly related and are greatly separated, phylogenetically speaking, as their divergence point is estimated at least 300 million years ago [1]. These two species have in common to be harmless, easy to grow and genetically tractable. Yet, while *S. cerevisiae* is commonly used in different kinds of industry (for example, brewery and bakery), which explains why it is a widely studied model, *S. pombe* use outside of the lab is way more limited. To understand why this species has also become a widely used model organism, its features have to be examined.

### **Life cycle**

*S. pombe* features are what made it a useful model organism. Among these features, its life cycle (**Figure 1**) is of major importance. It shows a typical eukaryotic life cycle, polarized growth pattern and defined shape. In this species, vegetative cells are haploid but, in case of nutritional stress, typically nitrogen starvation, two haploid cells undergo sexual differentiation and fuse together to form a single diploid cell that will undergo meiosis. The existence of both states (haploid and diploid) at clearly separated time is an asset for research, as both ploidy numbers and reproduction methods come with advantages and disadvantages. For example, in diploid organisms with sexual reproduction, the heterozygote status adds a difficulty to the analysis of mutation related phenotypes. In contrast, a fixed haploid state eases genetic studies, as only one allele is present for each gene, but meiosis cannot be studied and crosses cannot be used.

The entry into sexual reproduction is driven by external stimulus such as nutrient limitation and the presence of cells of opposite mating types. The mating type of a cell is determined by the gene expressed at the mating type locus. Switch between mating types inside a population is possible, provided that the strain carry both genetic information as it is the case for homothallic strains. The switch is carried out by epigenetic mechanisms that have been the first major subjects to be studied in *S. pombe*. The symbols “h<sup>90</sup>”, “h<sup>+</sup>” and “h<sup>-</sup>” historically refers to strains “Homothallic, yielding 90% sporulation”, “Heterothallic, Plus-Normal” and “Heterothallic, Minus Stable”. Heterothallic strains only carry one mating type cassette and thus are unable to perform mating type switching.

In conditions triggering sexual differentiation, cells will express pheromones in their immediate environment, which are specifically recognized by receptors of cells of the opposite mating type. In presence of a potential partner of the opposite mating type, two cells will perform conjugation to form a single diploid cell that will directly enter meiosis and form four haploid spores contained in an ascus: the tetrad. The fact that the product of one meiosis is gathered at

one location makes possible to analyze the full product of one meiosis at a time. This feature can be useful to prove the synthetic lethality of two mutations, for example. For a more extended view of *S. pombe* history and life cycle, see “Introduction to fission yeast as a model system” (Hayles and Nurse, 2018) [2,3].

### **Genetic features**

*S. pombe* is thus particularly useful to study cellular cycle, especially meiosis and cell differentiation. Moreover, it serves as a model for the study of RNA interference, chromatin modification, centromere dynamic, replication origin, splicing and small ncRNA regulation as these processes are more similar between *S. pombe* and mammals than they are between *S. cerevisiae* and mammals [4,5]. For example, *S. pombe* shares the characteristic epigenetic modifications of heterochromatin that are found in human [6,7]. Although differences obviously exist between the chromatin of fission yeast and mammals, for example a shorter inter-nucleosome repeat length or absence of linker H1 histones [7], it constitutes a better model than the budding yeast for epigenetic studies on processes conserved in mammals. For splicing also, about 50% of fission yeast genes contains at least one intron which is much more similar to human than the very low number of intron containing genes in budding yeast [2].

Finally, other advantages of using *S. pombe* as a model organism include a restricted number of chromosomes (three) and a relatively small genome (13.8 Mb). These characteristics are particularly important as they facilitate whole genome sequencing, as performed in the course of this master thesis, allowing *S. pombe* to be the sixth eukaryotic genome fully sequenced [1,2,8].

### **The stress response and the TOR pathway in fission yeast**

Unicellular organisms, as they have little control over their environment, have to rely on efficient stress response pathways to endure variation in nutrient availability. In the fission yeast, a lot of these pathways exist, and some of them also regulate sexual differentiation. Among those regulatory pathways are the TOR kinases. TOR (Target of rapamycin) is a widely conserved serine/threonine kinase. In fission yeast, there is two homologs of TOR contained in two complexes named TORC1 and TORC2. They regulate cell cycle and anabolic processes in response to nutrient availability. TORC1 and TORC2 play opposite roles in the regulation of these processes. TORC1 promotes cell proliferation and represses sexual differentiation under nitrogen rich-condition while TORC2 is required for induction of sexual differentiation. Among the substrates of TORC1, the protein S6K1 – Psk1<sup>1</sup> in *S. pombe* (p70 ribosomal protein S6 kinase 1) – is well described. Psk1 is a member of the AGC (protein kinase A/G/C) kinase family, which contains highly conserved enzymes that regulate growth by phosphorylating ribosomal protein S6 in response to nutrient availability [9,10].

Regulations directed by TORC1 is one of the pathway for which fission yeast is considered a good model, because partial inhibition of TORC1 by rapamycin does not inhibit growth of wild-type strain, as it is the case in most other eukaryotic cells. In addition, the presence of two independent TOR kinases within TORC1 and TORC2 (while in higher eukaryotes the TOR kinase is shared between complexes) is an asset to dissect the function of these two complexes [10].

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<sup>1</sup> In *S. pombe*, the convention is that the name of the protein is composed of three letters and a number. Wrote with a capital, it refers to the protein while wrote in italic it refers to the corresponding gene.

# The RNA polymerase III

## Importance in fundamental cellular function

Besides their obvious importance in translation, RNAs perform central roles in organisms, including maintenance, defense and regulation. First eukaryotic non-coding RNAs (ncRNAs), tRNAs and rRNAs, were discovered in the 50s while existence of other types of ncRNAs was not established until the 80s. These molecules regulate processes as general as transcription, translation and mRNA stability as well as fine tuning processes as splicing, gene silencing and molecular scaffolding [11].

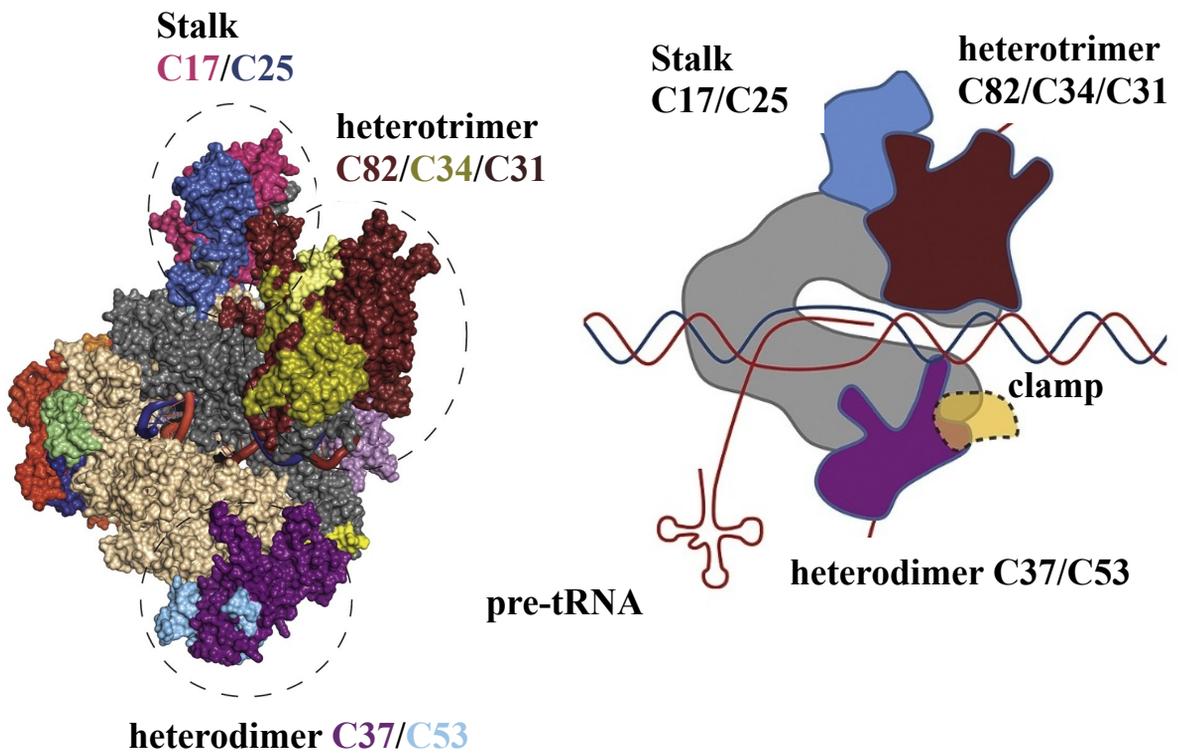
Nucleotidic DNA-dependant RNA polymerization in Eukaryotes is performed by several major complexes called RNA polymerases while in Prokaryotes and Archaea, the same burden is handled by a single complex [12,13]. In fission yeast, there is three of such complexes, each of them is specialized for a subset of transcripts: Pol I is responsible for the synthesis of ribosomal RNAs (rRNAs) while Pol II synthesizes messenger RNAs (mRNAs) and a set of non-coding RNAs (ncRNAs) and Pol III is in charge of the synthesis of transfer RNAs (tRNAs) and of a few other ncRNAs. These specializations come along with special features that tune the polymerase with the special requirement associated to its main type of transcript. For example, the high stability of Pol III transcription factor TFIIB allows a high rate of re-initiation (one re-initiation every 1.1 to 1.3 seconds) on the same template, thus improving its global transcription rate [14].

As the scientific community historically focused on protein coding genes, Pol II was by far the most studied of the three RNA polymerases. However, Pol III has experienced a renewed interest lately due to the discovery of many new roles for tRNAs including implication in tumorigenesis. Among those implications, we can cite the involvement of a tRNA processing enzyme in prostate cancer, the oncogenic impact of codon misreading by tRNAs and tRNA modification enzymes as genome stability warrants [15–17]. But Pol III is also implicated in a lot of different cellular processes through the synthesis of tRNAs, but also of other transcripts: the 5S rRNA and ribonucleotidic element of the spliceosome, of the recognition particle and of RNase P [18–20]. Those transcripts make Pol III also necessary for intron splicing and protein localization.

## Structure

RNA polymerases are very ancient structures that undergo global alteration during the course of evolution. The prokaryotic RNA polymerase is composed of only four subunits while eukaryotic RNA polymerases are large complexes consisting of at least twelve subunits for a molecular mass over 500 kD. Archaeal polymerases are closer to eukaryotic, as they are composed of the core bacterial polymerase homologs and additional subunits recruited for a total number of twelve subunits. Eukaryotic RNA polymerases have homologs of all eleven archaeal RNA polymerase subunits plus an homolog of an archaeal general transcription factor (GTF) [13,21].

Between them, eukaryotic polymerases share common features but also big differences. For example, Pol III has five additional subunits to Pol II that form two additional subcomplexes,



**Figure 2: Pol III structure in Cryo-EM.** Detailed (left) and schematic (right) structures of the RNA polymerase III in open conformation. The stalk and the two subcomplexes with no homologue among Pol II subunits are highlighted, with their corresponding subunits. Transcribed DNA, with template strand (blue) and non-template strand (red) is represented [120].

the C37/C53<sup>2</sup> heterodimer and the C82/C34/C31 heterotrimer, for a total of 17 subunits [22] (**Figure 2**). Transcription by Pol III also requires far less GTFs than by Pol II. GTFs are proteins and multiprotein complexes that are required for transcription *in vivo* and differ between the three polymerases except for Transcription Factor B (TFB) and TATA-binding protein (TBP) that are common. A conceivable scenario that explains both the subunit gain and the GTF loss of Pol III is that these GTFs were recruited through evolution to serve as constitutive subunits for the complex [13]. This theory comes from the fact that the two Pol III additional subcomplexes are structural and functional homologs to the Pol II GTFs TFIIE and TFIIIF. Evolution of GTF into polymerase subunits is not exclusive to Pol III as it also concerns Pol I and seems to have also occurred to the ancestral eukaryotic polymerase [23].

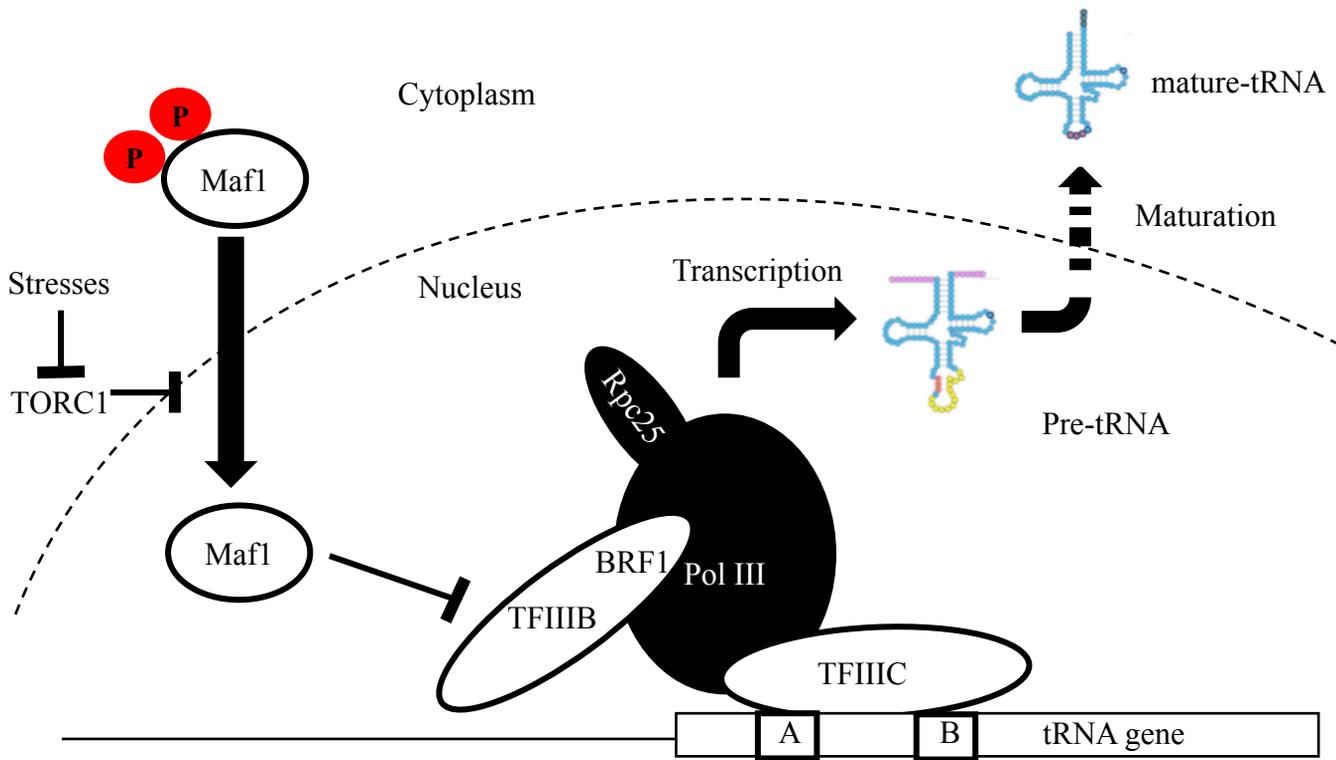
## **Rpc25**

Previous work from the laboratory revealed by serendipity that the addition of a small peptide (the flag) at the C-terminal end of a subunit of the RNA polymerase III (Pol III), Rpc25, leads to a marked defect in pre-tRNA production and an impaired growth in the resulting strain, *rpc25-flag*. Rpc25 is an essential Pol III subunit located at the surface of the complex. Phylogenetical analysis and complementation assay have shown that it is a paralog of the Rpb7 subunit of Pol II. It is also very conserved in non-bacterial RNA polymerases [24]. It forms a heterodimer with Rpc17, another essential subunit that binds the TFIIIB subunit Brf1. This heterodimer (C17/C25) corresponds to the Pol III stalk [25], whose exact function remains unknown but that is conserved in Pol I and Pol II. However, previous studies focusing on the phenotype of different *rpc25* mutants have been performed, leading to precious insight into what kind of function could be impacted by the flag addition.

In *Saccharomyces cerevisiae*, mutation of Rpc25 conserved residue S100 impairs the *in vivo* synthesis of Pol III transcripts, as well as *in vitro* synthesis, leading to a growth defect at 37 °C. However, *in vitro* studies of tRNA synthesis showed that the mutation does not affect transcription elongation or termination. Instead, this subunit seems to be important for the initiation step of transcription as shown by the fact that, *in vitro*, the defect can be relieved by pre-assembly of the initiation complex. But the authors of that study could not exclude a defect in the assembly or the stability of the complex [26].

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<sup>2</sup> In *S. pombe*, all RNA polymerase subunits are annotated as “RpX” where “Rp” refers to RNA polymerase and X is a letter corresponding to the complex that includes the subunit, A for pol I, B for Pol II and C for Pol III. These 3 letters are followed by the number that refers to a specific subunit. Often abbreviated as only the last letter and the number as in “C84”.



**Figure 3: An overview of Pol III initiation.** Pol III pre-initiation complex is composed of Pol III, TFIIB and TFIIC, which binds the A and B boxes located inside Pol III transcribed genes. Upon stress, TORC1 stop phosphorylating Maf1. Unphosphorylated Maf1 enters the nucleus and impairs the TFIIB-dependent Pol III recruitment, causing Pol III transcription inhibition. Brf1 is a subunit of TFIIB and Rpc25 is a subunit of Pol III [55].

## Transcription initiation

Despite subunits differences, all three polymerases share a common process for initiating the transcription. First, a Pre-Initiation Complex (PIC) is formed at the transcribed gene promoter by recruitment of the polymerase to its target gene. This recruitment is dependent on a set of transcription factors that depend on the polymerase, although some components are shared, for example TBP. At this point, the polymerase complex is in a closed state conformation. Then, the double-stranded DNA in this complex is melted, forming the transcription bubble inside the now opened complex [27].

The Pol III PIC (**Figure 3**) formation only requires two transcription factors on the majority of its transcriptome: TFIIB and TFIIC. Prior to transcription, TFIIC first binds the internal promoter of Pol III transcribed genes, allowing TFIIB recruitment to the upstream DNA. Contrary to Pol II-transcribed genes, most of Pol III-transcribed genes lack any upstream regulatory elements. The transcription factors binding sequences are located within the gene. TFIIB is responsible for Pol III recruitment and transcription initiation and is composed of three subunits: TBP (TATA-box Binding Protein), Bdp1 (B double prime 1) and Brf1 (TFIIB-related factor) [28]. In the exclusive case of *snu6*, a snRNA gene that encodes the U6 snRNA which is a part of the spliceosome, TFIIB alone is sufficient to promote Pol III transcription. In this specific case, the TFIIB subunit TBP binds the DNA at the TATA-box located upstream of the promoter [29].

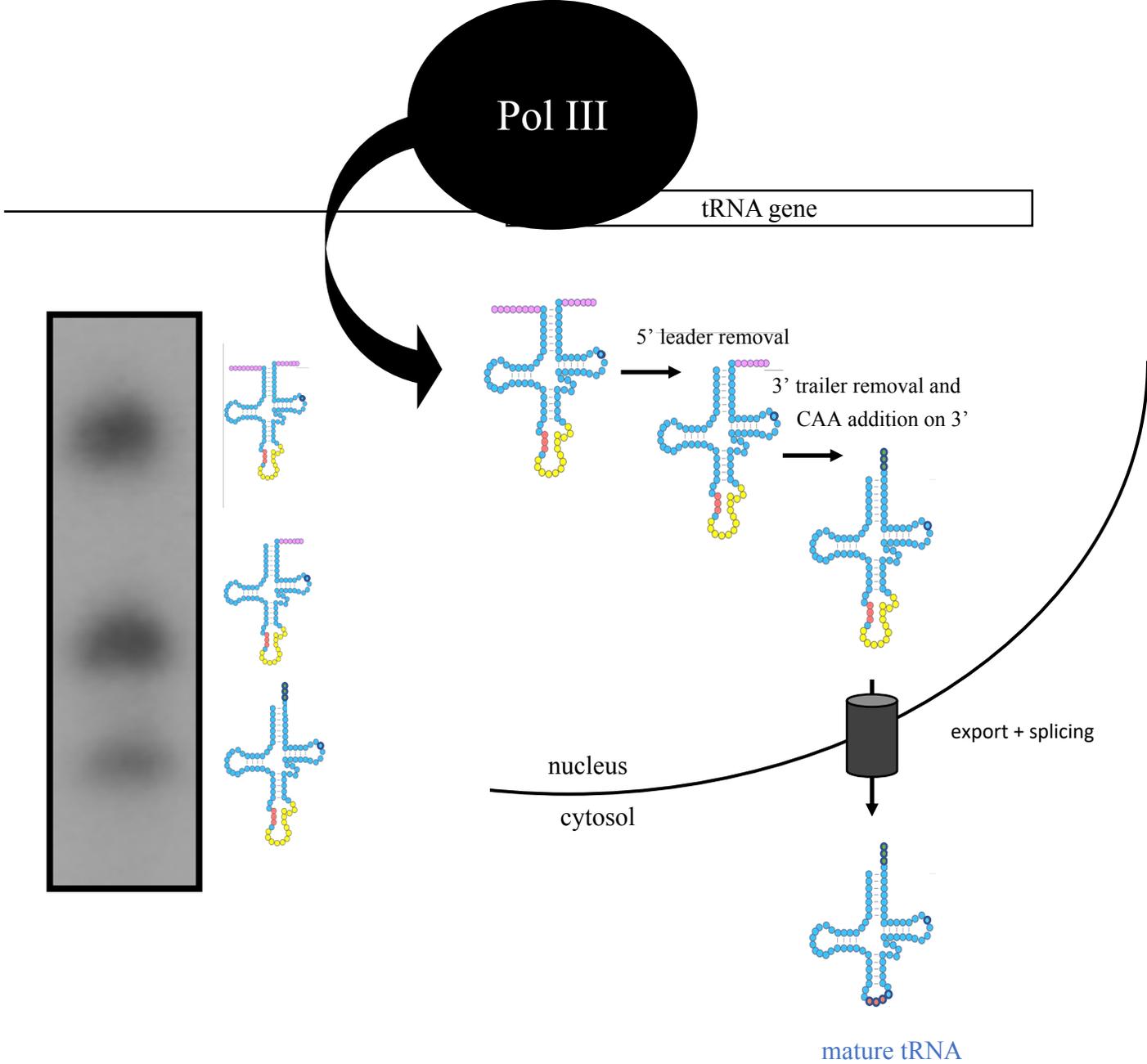
If TFIIB and TFIIC are sufficient, together with Pol III, to initiate transcription of tRNA gene *in vitro*, a third transcription factor, TFIIA, is specifically required for the transcription of 5s rRNA. It binds the RDN5-specific C box that is specific to this gene and recruit TFIIC [30]. In contrast to the six general transcription factors of Pol II, TFIIA, TFIIB and TFIIC represent the complete list of known Pol III transcription factors [28].

## Regulation of Pol III

The only known protein responsible for regulation of Pol III transcription in fission yeast is Maf1. The Maf1 protein prevents the recruitment of Pol III to its target promoters [31] but the exact mechanism by which it inhibits transcription is still discussed. Some studies suggest that Maf1 binds the C82-C34-C31 heterotrimer and causes a rearrangement that loosen its interaction to Brf1, other studies suggest that it binds Brf1. Finally it could as well binds both protein [32,33]. Anyway, the binding of Maf1 to Pol III transcription machinery is dependent upon nuclear targeting of this protein by NLS (Nuclear localization signal), which requires Maf1 to be dephosphorylated as phosphorylation cause the NLS sequence to be masked [34]. This phosphorylation is the most characterized way to regulate Pol III transcription.

Regulating phosphorylation of Maf1 take place at PKA phosphorylation sites [35]. Several central signaling pathways, including TOR, Pkc1 and CK2, link a variety of external and internal cues (nutrient availability, DNA damage, cellular integrity...) to Maf1 phosphorylation and thus, to the activity of Pol III [35,36]. This regulation mode likely conserved, as Maf1 orthologs act as Pol III repressors in mammals and plants as well [37,38].

Other regulation sources for Pol III described in other organisms than *S. pombe* include protein modifications occurring on Pol III subunits, like sumoylation of Rpc82 [39], or on its TF, like



**Figure 4: Steps of pre-tRNA processing and their correspondence in Northern Blot.** On the left, the premature forms of tRNA Arg in a WT strain can be discriminated based on their size as shown on a Northern blot. On the right, the processing steps of tRNA maturation are shown: the removal of the 5' leader, the removal of the 3' trailer and addition of CAA on 3' end, the export outside of the nucleus and the splicing of the introns. See Hopper et al., 2003 for a more detailed view [47].

phosphorylation of Bdp1. This last protein modification is also directed by a TORC1 dependent pathway [40]. Other proteins, including Nab2 a polyadenylated RNA-binding protein implicated in the maturation and export of tRNA [41], or the retinoblastoma tumor suppressor protein in human [42], are also implicated in this process. However, the existence and activity of these sources in *S. pombe* is still to demonstrate.

### Processing of pre-tRNA

Pol III most numerous transcripts, tRNAs are also regulated post-transcriptionally and independently from Pol III. Pol III never directly produces matures tRNA, they are derived from pre-tRNA through numerous events of modification and splicing that are either common to all tRNA or more specific. These steps present opportunities for regulation allowing a fine tuning between tRNA content within the cell and translation requirement. Such regulation occurring at many different points of the maturation process can lead to the occurrence of buffering effect affecting the ratio of pre-tRNA on mature tRNA. For example, deletion of *mafl* in mouse causes an increase in Pol III activity and thus an excessive level of pre-tRNA, but mature tRNA level is not impacted [43]. As well, in a wide variety of mutants with altered pre-tRNA content, level of mature tRNAs is less affected by the mutation than the level of pre-tRNA [44].

tRNA subcellular trafficking and modifications are regulated at different steps by many external stresses [45,46]. Reversely, tRNA status within the nucleus seems to be somehow reported to the translation machinery and to other process within the cell, for example stress response [47]. Recently, a link between pre-tRNA level and the sexual differentiation of *Schizosaccharomyces pombe* has been demonstrated. Mutants lacking enzymes implicated in the tRNA maturation and processing (e.g. aminoacyl-tRNA synthetase) show an inhibition of the TORC1 signaling pathway, leading to ectopic initiation of the sexual differentiation program [48].

The three steps common to all tRNAs are: removal of the 5' leader by RNase P, removal of the 3' trailer and addition of the trinucleotide CCA in 3' (**Figure 4**). The maturation of some tRNA will also require introns splicing steps. Besides, tRNA undergoes numerous specific modifications including methylation, pseudouridylation or deamination. These modifications can take place in the cytoplasm or in the nucleus [47,49]. They involve numerous enzymes that overexpression can either increase, decrease or let unimpacted the global level of mature tRNA within the cell. Among these proteins, Sla1 overexpression is known to tremendously increase the level of pre-tRNA [48].

Sla1 is the ortholog of the human La protein. The La protein is an abundant nuclear phosphoprotein that acts as an RNA chaperone. It binds newly synthesized pol III transcript, including pre-tRNA, precursor of the 5SrRNA and U6 snRNA. The binding site to this protein is localized at the 3' end of precursor RNA. It facilitate pre-tRNA 3' maturation through stabilization of these molecules in a conformation that eases endonucleolytic cleavage [50].

Despite regulation of tRNAs an important and highly ramified process, a subset of tRNAs seems to partially escape this process. These tRNAs show low responsive to environmental or cellular changes that lead to a stress condition. In this subset, at least one tRNA for every amino acid is found, suggesting that it represents a set of housekeeping tRNA that have the purpose to keep a minimal level of translation ongoing, no matter what [51]. Levels of tRNAs also affect protein folding by impacting translation speed. In normal conditions, rare codons are used to encode specific parts of the protein (N-terminal end, unstructured regions, signal peptides...)

as they slow down translation. In eukaryotes, this effect is mainly studied because silent mutations in codons can affect co-translational protein folding and lead to diseases [52].

## Chromatin and histone modifications

### Common features of chromatin

A major factor impacting RNA polymerases function is the chromatin state of their target genes. This is true for Pol II but also for Pol III, with some features specific to each polymerase. In the context of this study, some of the actors responsible for chromatin modifications (i.e. SAGA, Pol II or actors recruited by Pol II) are implicated on potential regulation of Pol III.

The chromatin state corresponds to the variable packaging that surrounds DNA within the nucleus. This state is determined by the modifications added to histones, assembled into octamer around which the DNA is wrapped. Each histone is composed of a main globular core and of a N-terminal tail that acquires most of the modifications. The most common modifications are methylation, phosphorylation and acetylation but other modifications, for example ubiquitination, can be encountered. Moreover, some modifications can be present at different position on the histone tails and in a different number. All of these histone modifications provide a wide range of possible regulation sources for transcription [53].

The two characterized mechanisms for the effect of histone tails modifications to pass on to the global chromatin state are disruption of contacts between histones, causing an untangling of the DNA wrapped around them, and recruitment of other proteins. The second mechanism causes a subset of protein to be recruited depending on the modifications and that this subset of protein itself has an effect [53]. However, the first function impact is also of huge importance and can have a widespread effect on transcription. For example, lysine acetylation generally promotes nucleosome eviction, notably through charge neutralization of the residues they are attached to, which unwraps the DNA and increases the processivity of Pol II in transcription elongation phase [54].

### Chromatin at Pol III genes

Some chromatin features characteristic of active genes are shared between Pol II and Pol III while others are specific to one of the polymerase area of transcript. Shared histone modifications include H3K4/9/23/27<sup>3</sup> acetylation and H3K4 methylation correlating with apparent activity of the gene as well as H3K9 tri-methylation and H3K27 di and tri-methylation correlating with apparent inactivity. On the other hand, some chromatin modifications generally found in Pol II transcribed regions are absent from Pol III-transcribed genes. These features include H3K36 tri-methylation and H3K79 di-methylation in mammals [55,56].

As chromatin modifications influence transcription, the reverse assertion is also true. Pol III transcribed genes also influence the chromatin state of their neighborhood gene as active t-

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<sup>3</sup> HX will always referred to the histone type. First the type of histones, then the type of amino-acid affected and its position among similar amino-acid and to conclude the modification that affects this amino acid, in an abbreviate form, “me” for methylation, “ac” for acetylation, “ub” for ubiquitination and so on. For example, H3K4me refers to a methylation of the fourth lysine of a type three histone.

acetyltransferase recruitment and on nucleosome depletion at specific loci and also on the function of TFIIC linked to cohesion. TFIIC can also accomplish this function in loci containing no Pol III transcribed genes [58,59].

## Pol II link with Pol III

Results provided in this Master Thesis presented hereafter indicate genetic and functional interactions between transcription performed by Pol III and Pol II related proteins, as well as with Pol II itself. Other insights into a trans-polymerase regulation have been obtained previously in different kind of studies performed in a range of organism ranging from yeast to mammals. In particular, high throughput sequencing related techniques provided clues of the implication of Pol II in the regulation of Pol III [60].

First insights of a Pol II dependent regulation of Pol III transcription came from the fact that specific inhibition of Pol II by alpha-amanitin affects the transcription of a subset of Pol III-transcribed genes [61]. However, it was hard to conclude anything from these effects because suppression of Pol II transcription rapidly impacts a tremendous number of cellular processes. Thus, this correlation alone was not sufficient to assert a functional relationship between the two processes [60]. Later on, ChIP-Seq<sup>4</sup> analyses revealed Pol II occupancy at Pol III-transcribed genes in human cell lines and also in mouse and drosophila [55]. Unpublished data from our lab (Carlo Yague-Sanz, PhD in progress) expanded these findings to fission yeast *Schizosaccharomyces pombe* (*S. pombe*)[62].

Recent technical advances led to additional insights of this potential relationship between the two polymerases. In particular, MNase-seq<sup>5</sup> revealed that in fission yeast, mutation of the Pol II largest subunit strongly affects nucleosome occupancy at Pol III transcribed genes [63]. This mutation concerns the Rpb1 C-terminal domain, most commonly referred to as the CTD, which will be more thoroughly described in a dedicated section below.

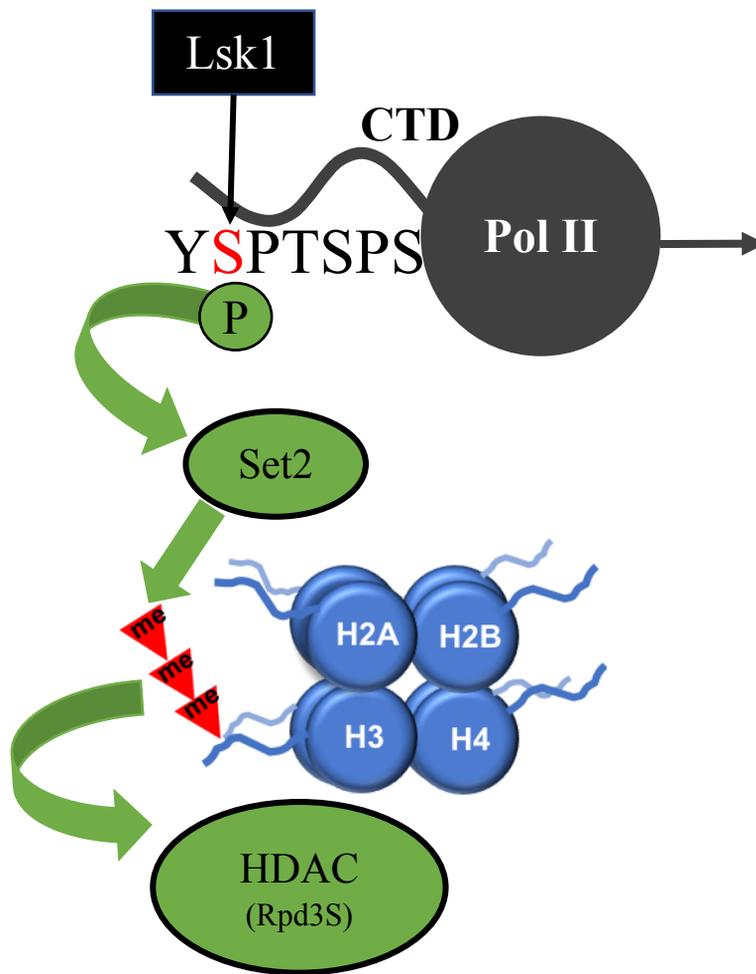
Pol II active transcription can influence Pol III transcription by affecting chromatin as read-through by RNA polymerase impacts nucleosomes located on the transcribed sequence. As previous work in our laboratory detected Pol II presence at Pol III transcribed sites, and numerous Pol II transcribed sites are located antisense to Pol III transcribed genes, this could also impact chromatin state at Pol III transcribed genes. Moreover, Pol II is not only present at Pol III transcribed gene but is also transcriptionally active at these sites, as indicated by the fact that its CTD is phosphorylated at serine 2 and 5, which are modifications specific to Pol II elongation and initiation respectively [6,18,55].

The emerging hypothesis is thus that Pol II transcription might facilitate Pol III transcription by modifying the local chromatin structure at these locations, while the Pol II-dependent modifications at Pol III transcribed genes are strongly supported by the fact that most of the modifications characteristic of euchromatin are shared between active Pol II and Pol III transcribed genes and that, on the U6 snDNA, H3K4 acetylation is dependent on Pol II [64].

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<sup>4</sup> ChIP-Seq is an assay in which the input molecule is DNA crosslinked to a specific protein that has been immunoprecipitated. The position of this protein on the sequence is inferred from the sequence of the DNA that is precipitate with it.

<sup>5</sup> MNase-seq is an assay in which the input molecule is DNA derived from micrococcal nuclease digestion. Nucleosome position on the sequence is determined from their ability to protect DNA from digestion by MNase



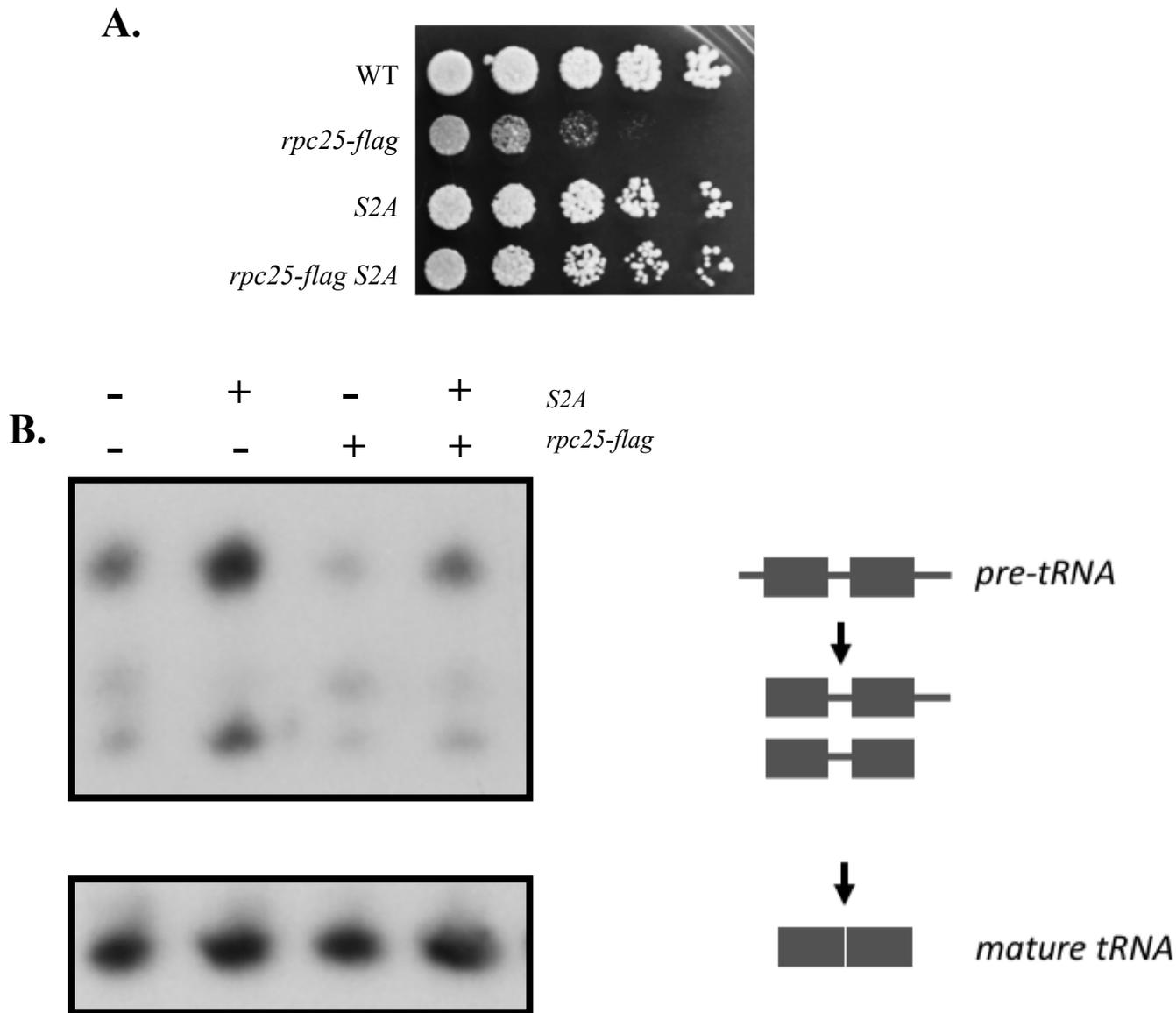
**Figure 5: Principle of transcription regulation by the CTD.** Modifications of CTD residues of the repeated heptamer (here only phosphorylation on Serines 2) induce the recruitment of proteins (here Set2). Some of them modify chromatin (here tri-methylation on H3K36) that will, in turn, recruit other proteins (here, Histone DeAcetylase (HDAC)) [66] .

## The Rpb1 C-terminal domain (CTD)

The CTD is a major platform for the co-transcriptional recruitment of chromatin modification enzymes, transcription regulatory factors, elongation factors, termination factors and many other proteins. It is a central element for the regulation of RNA synthesis by Pol II. The CTD length is highly variable between species, but its main features are highly conserved, including the consensus sequence that is repeated a variable number of times. This consensus sequence is composed of a repetition of seven amino acid (YSPTSPS) that can be heavily modified, phosphorylated, glycosylated or isomerized, its structural plasticity enabling different interaction with other proteins depending on its modification state. In particular, the three serines (S2, S5 and S7) of this repeated sequence are phosphorylated and dephosphorylated in a transcription-dependent manner [65].

Modifications of residues composing this consensus sequence (for example, phosphorylation) lead to, or instead impede the recruitment of proteins. These proteins or the modifications they execute can also lead to the recruitment of other proteins that will, in their turn, induce modifications or other effects. This kind of cascading effect leads to a fine-tuning in regulation of co-transcription processes along the Pol II transcribed gene and is critical, in particular, for co-transcriptional processing of the pre-mRNA.

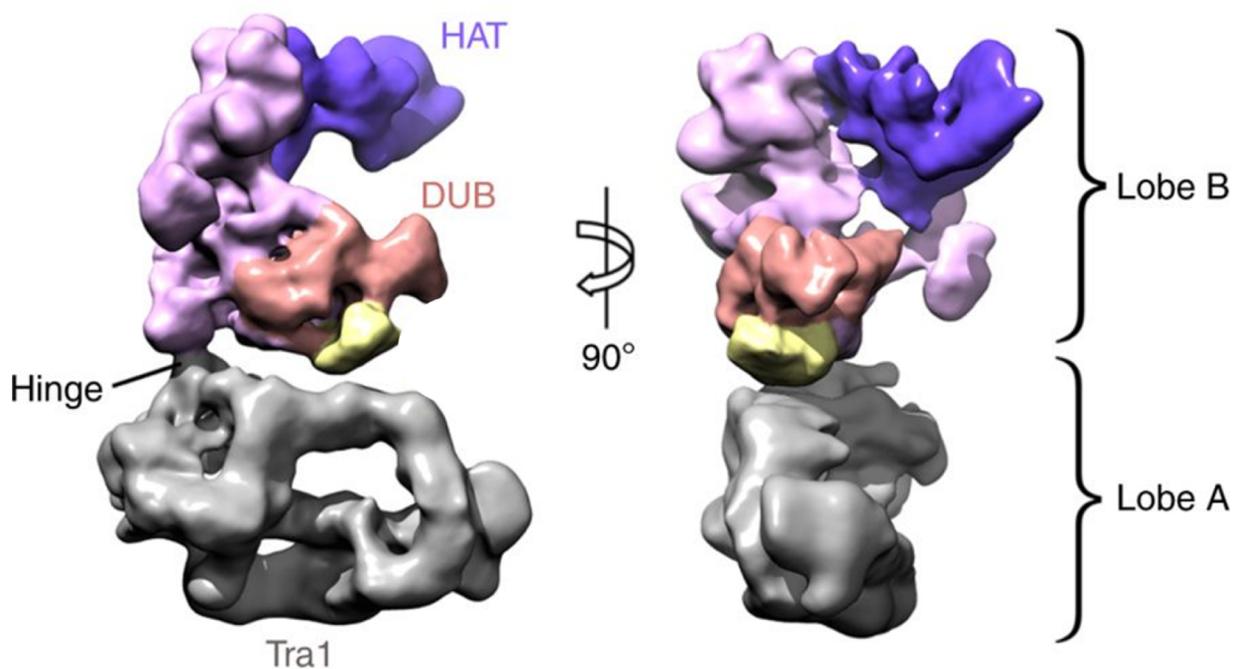
In our context, we will focus on a particular modification of the CTD: the phosphorylation of the serine in position 2 (referred as S2). This phosphorylation occurs during the elongation phase of the transcription, peaking at the 3' end of the gene, close to the polyadenylation signal. It is required for the H3K36 methylation through the recruitment of the Set2 methyltransferase, which leads to the recruitment of other enzymes such as the histone deacetylase Rpd3S, resulting in a localized change of chromatin state depending on the polymerase transcription phase [66]. Phosphorylation of S2 is performed in fission yeast by the enzyme Lsk1 (Cdk12) (**Figure 5**), which is regulated in response to stress conditions by phosphorylation by the Sty1 MAPK downstream to the Wis1 MAPKK. This pathway allows regulation of transcription by Pol II in response to stress conditions and also participates in the regulation of the initiation of sexual differentiation [67].



**Figure 6: Phenotype of the *S2A rpc25-flag* double mutants.** A) Spot dilution assay with serial dilutions of the wild type (WT) and the indicated mutants strains on YES medium. Plates were incubated for 72 hours at 32°C B) Northern blot analyses of pre-tRNA content in WT, *rpc25-flag*, *S2A* and *rpc25-flag S2A* mutants. Strains were grown in liquid YES medium at 32 °C until mid-exponential phase (O.D 0.5). RNA extracts were analysed by northern blot with a probe against SPBTRNAARG.05. Schematic representation of the observable form of pre-tRNA are showed on the right. Results from C. Yague-Sanz.

In fission yeast (as it is the case in other species [68]) mutants where the CTD S2 phosphorylation is abolished (through mutation of the CTD S2 kinase *Lsk1* :  $\Delta$ *lsk1* [69] ; or mutation of every serines S2 into unphosphorylatable alanine: CTD *S2A*) are viable. They show altered patterns of histone methylation and acetylation, leading to differences in nucleosome dynamics[63]) and global phenotype as decreased mating efficiency and growth [67,70]. Strikingly, Pol III transcribed genes were among the most affected regions in the CTD *S2A* mutant, suggesting once again that the presence of Pol II at Pol III transcribed genes is biologically relevant, maybe by influencing chromatin state and histone modifications at these loci [63]. Moreover, the CTD *S2A* and the *lsk1* deletion mutant display increased pre-tRNA production (results from Carlo-Yague-Sanz), suggesting a negative regulation of Pol III arising from this modification of Pol II independently from Maf1.

Finally, one last insight in a Pol II-dependent regulation of Pol III came from results precluding to this master thesis. A hypomorphic mutant of the Pol III subunit *Rpc25* displays a reduced amount of pre-tRNA and a slower growth and, surprisingly, both phenotypes can be suppressed by mutants where the CTD S2 phosphorylation is abolished (**Figure 6**). This genetic interaction further strengthens the connection between Pol II CTD phosphorylation with the regulation of the expression of the Pol III-transcribed tRNAs.



**Figure 7: Structure of SAGA.** 3D structure of the SAGA complex from two different angles. The TF binding module (Tra1) is colored in grey, the Histone Acetyl Transferase (HAT) module in purple, the DeUBiquitylation (DUB) module in orange, and the structural core in pink and TBP binding module in yellow [74].

## SAGA

Among the regulators of Pol II that could play a role in the regulation of Pol III, the SAGA complex is of major interest according to the preliminary results of this master thesis. SAGA (Spt-Ada-Gcn5 acetyltransferase) is an evolutionary conserved transcriptional co-activator of Pol II comprising 18 to 20 subunits, depending on the species. Co-activators are directly implicated in transcription but, in contrast with GTFs, they are not compulsory for transcription *in vivo*. The SAGA complex is recruiting TBP at its target genes and also performing acetylation (on H3K9 mainly) and deubiquitylation (on monoubiquitylated H2B) on histones which leads to the opening of the chromatin structure at promoters [71,72]. These subunits are organized in modules depending on their main function: structure, histone acetyltransferase, deubiquitylation or activator binding (**Figure 7**) [73].

Structurally speaking, the SAGA complex is composed of two lobes linked together by a hinge. The A lobe is composed of one single 430 kDa protein, Tra1. The B lobe contains the other proteins which are responsible for the catalytic activities; DUB and acetyltransferase [74]. The structure of SAGA induces cascading effects emerging when subunits of the complex are depleted. For example, among the DUB module that is composed of the subunits Ubp8, Sgf11, Sus1 and Sgf73, depletion of Ubp8 causes the loss of two other subunits of the DUB module (Sgf11 and Sus1) and reversely, depletion of Sgf11 or Sus1 results in the loss of the two other proteins. Loss of Sgf73 is causing the detachment of every other subunits of the DUB module as it is anchoring the module into SAGA through its C-terminal part [54,75].

The largest subunit of SAGA is Tra1, which defines by itself one of the two lobes of the complex. Tra1 belongs to the PIKK (PI3 K-like kinase) family but does not have any catalytical activity. Instead, it has a role in scaffolding [76] and regulation of the SAGA complex. The regulation by Tra1 passes by its ancient catalytic center (i.e. the conserved domain where the kinase activity takes place in the other proteins of the PIKK family), although Tra1 has lost its activity. This loss of activity let this site wide open for the binding of regulatory factors. Protein binding in this enlarged cavity results in a modification of Tra1 conformation that is transmitted through the hinge, finally affecting the whole SAGA complex and in particular the activity of the catalytic subunits, located in the other lobe [74]. The deletion of *tra1* is lethal in mouse and *S. cerevisiae* because it is also a part of the essential NuA4 complex, but viable in *S. pombe* where a paralog, Tra2, is responsible for its essential role within the NuA4 complex. Within SAGA, Tra1 is also implicated in the recruitment of the DUB module [77].

The activity of SAGA has a positive impact on transcription at a global level. Recent studies have showed that it is not restrained to a small subset (about 10 %) of genes, as proposed in seminal studies, but that mutations within SAGA affect most of the Pol II transcribed genes. However, the change in mRNA production resulting from mutations within SAGA is countered by an increase in mRNA stability, resulting in a global buffering effect. This, combined with the very dynamics interactions of SAGA with chromatin that makes its chromatin detection difficult, explain why SAGA was believed to be present at only a small subset of genes [71]. Interestingly, while it is described as a Pol II-specific co-activator, SAGA is found at tRNA genes in drosophila [78].

Proteins outside of SAGA subunits are also required for its function as recruitment of SAGA to its target genes is performed by Atf1 and Pcr1[79]. These are trans-activator proteins, which means that they bind DNA and recruit co-activator complexes (in this case, SAGA). Their

activity is regulated by cellular cues through, in particular, the MAP kinase pathway [80]. They serve as a bridge between cellular state and epigenetic modifications affecting transcription.

# *Material and Methods*

## **Biological material**

Our strain of great biological interest, *rpc25-flag*, results from the addition of a 1.6kb sequence right before the stop codon of the *rpc25* gene. The inserted sequence contains a linker, the flag sequence (about 150 bp) ending with a stop codon and followed by a gene encoding resistance to either hygromycin (*hphR*) or nourseothricin (*natR*) with its own promoter. This genetic manipulation was meant to create a strain in which Pol III could be immunoprecipitated but results in two unexpected phenotypes: a leaky temperature-dependent growth defect, with no visible growth at 37 °C, and a decreased concentration in pre-tRNA within the cell. This difference also exists for mature tRNA, but at a reduced level. The *rpc1-TAP* strain results from the addition of a TAP tag on the C-terminal end of the biggest Pol III subunit. The TAP is followed by a nourseothricin resistance gene with its own promoter. This strain was received from F. Bachand.

Some other strains used in this study were provided by other laboratories, in particular the deletion mutants used for the creation of potentially suppressive double mutants. The strains *Δsty1* and *Δpcr1* were provided by E. Hidalgo, *Δgcn5*, *Δsgf11* and *Δada2* were provided by D. Helmlinger. And finally the strain *psk1-13Myc* was provided by F. Tamanoi.

## **Growth assay**

Spot dilution assays were performed in order to determine the presence or absence of a temperature sensitive phenotype of growth defect. *S. pombe* cultures are grown in liquid YES (yeast extract supplemented) media at 32 °C until exponential phase (Optical Density (OD) at 0.595 nm= 0.5) from which 5-fold dilutions series are made with liquid YES<sup>6</sup> under sterile hood. For experiments concerning transformed strains with repression by thiamine, cultures are kept in repressive media (EMM<sup>7</sup>-leu + thiamine), then switched to inductive media (EMM - leu) at least 20 hours before spot dilution. 4,5µl drops from these dilutions are transferred on agar plates with a multichannel pipette and incubated at 25°C, 32°C and 37°C for 72 hours or 96 hours in the case of growth on EMM-agar.

## **Generation of double mutant**

Many different strains were produced during this master thesis, for example *rpc1-TAP rpc25-flag*, most of them by inter-breeding of already existing mutant strains. Crosses between an *h*-strain and an *h*+ strain were performed on malt extract plates at 25 °C for 48 hours, in order to induce sexual differentiation and mating. After assessment of the presence of ascospores, 1 ml of sterile water was inoculated with a full loop of the cross result. 2 µl of β-glucuronidase/Arylsulfatase (Sigma-Aldrich) are added to the solution in order to digest asci walls. After at least 6 hours of incubation at 29 °C with agitation or incubation overnight at 4°C, the product was washed in SDS 1% to get rid of the remaining intact cells and resuspended

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<sup>6</sup> Yeast Extract Supplemented, rich medium

<sup>7</sup> Edinburg Minimal Media, defined medium

in water. The efficiency of the digestion (absence of intact cells) is roughly assessed under a photonic microscope. Appropriate dilutions, generally around one hundred times, of the spores were spread on YES agar plate. After two to four days of incubation at 25 °C, the plates were replicated on selective media and, after two more days, some surviving colonies were isolated.

The product of  $\beta$ -glucuronidase digestion of the ascospore can be kept at 4°C for at least a month. As the two mutated alleles are often located on the same chromosome (there is only three nuclear chromosomes in *S. pombe*), the obtention of some double mutants requires an appropriate crossing-over between parental chromosomes. Therefore, in several cases, random spore analysis had to be performed multiple time until a double mutant was isolated.

After presumptive double mutants have been isolated and selected on the antibiotic containing media and prior to any further experiments, these strains were checked for the presence of the allele *rpc25-flag*, for their mating type and also for the presence of the other parental strain mutation by PCR and by sequencing of PCR products for *rpc25-flag* in *rpc1-TAP rpc25-flag* only. They were also checked for the Rpc25-flag presence at protein level by Western Blot. Mating type PCR allows the assessment of the mating type of the strain, as well as eviction of diploid strains from the candidate pool. Strains that were negative for either of the mutation by PCR or Western Blot were evicted as well.

### **PCR and sequencing**

Hot start colony PCR were performed in order to characterize double mutants and suppressors genotype. Few cells from a *S. pombe* colony were taken with a sterile tip and put on a 100  $\mu$ l mix containing water, GoTaq Reaction buffer for PCR (Promega), dNTP and PCR probes (see **Table S1** for probe sequences). Cells were lysed by 10 minutes incubation at 100 °C then the GoTaq polymerase was added and activated during 4 minutes at 94°C. The samples were subjected to 35 PCR cycles with an elongation time of 1.5 minute at 72°C and annealing temperature of 51 °C in a Biorad 100 Thermal cycler. To assess the presence/absence of amplification, 20  $\mu$ l of the PCR products were visualized on a 1% agarose gel stained with ethidium bromide and run during 25 minutes at 140 V. The gel was revealed by UV exposure in an UVP BioDoc-It<sup>2</sup> Imager.

Sequencing of the PCR products was performed by Eurofin after silica-membrane-based purification (QIAquick PCR purification Kit © QIAGEN) following the manufacturer's instructions.

### **Western blot**

Alkaline protein extraction was performed as described in Matsuo et al., 2006 [81] from  $5.3 \times 10^7$  exponential phase cells in 10 ml of YES media. Trichloroacetic acid (TCA) extraction protocol was used instead of alkaline extraction when better protein quality was needed, for quantification of Rpc1-TAP and of phosphorylated Psk1 especially. For this last kind of protein extraction,  $8 \times 10^7$  exponential phase cells were harvested in YES containing 10% TCA. After resuspension in water containing 10 % TCA, cells were broken by zirconium beads in a FastPREP-24 Classic Instrument (mpbio), then separated from the beads by centrifugation through a pierced Eppendorf. After the extraction, purified proteins were resuspended in a Tris HCL, SDS, glycerol, Bromophenol blue and  $\beta$ -mercaptoethanol Buffer. In the case of

quantification of Rpc1-TAP, the total amount of protein extracted was quantified by the classical Bradford method [82].

Afterwards, an equal amount of protein diluted in water for a total volume of 15 µl from alkaline extraction, or 15 µg of total protein extract from TCA extraction was charged for each condition on a 4-15% gradient polyacrylamide gel. After migrating for 40 minutes at 150 V, proteins were transferred to a membrane using a Trans-Blot Turbo Transfer System. The membrane was then blocked in PBS-Tween with 5% milk and incubated with the appropriate antibody (See Table S2) for 1 hour. Secondary antibodies used were the anti-mouse IgG antibodies covalently bound to horseradish peroxidase and the revelation was made by chemiluminescence after addition of ECL (entry-level peroxidase substrate for enhanced chemiluminescence).

In the case of phospho-Psk1 quantification, as phospho-Psk1 and Tubulin are migrating at the same position on a 4-15% polyacrylamide gel, the membranes were stripped between revelation of phospho-Psk1 and incubation with anti-Tubulin primary antibody. Stripping was performed by 30 minutes incubation at 55°C in a mercapto-ethanol, sodium dodecyl sulfate and Tris HCL buffer followed by washing in phosphate Buffer saline (PBS) 0.5% Tween. The membrane was then immersed in blocking buffer (PBS-Tween 0.5%+ 5% dehydrated milk) for one hour.

### **Suppressor isolation**

The protocol used to isolate suppressors for the temperature sensitivity phenotype is inspired from method used by Marinova and colleagues [83]. The mutant strain *rpc25-flag* was grown at the semi-permissive temperature (slight growth defect) of 32°C in liquid YES until exponential phase. This culture is then diluted 200 folds in 11 Erlenmeyers containing a total volume of 5 ml of liquid YES and in 94 wells of a 96-wells Masterblock plate, each one containing a total volume of 500µl. The two remaining wells are filled with liquid YES as a control against contamination. The same steps were performed with a wild-type strain as a control, but only one Erlenmeyer was filled. The 96-wells plate and Erlenmeyers were placed at a restrictive temperature of 37 °C with agitation.

After 72 hours, 10 µl were taken from the Erlenmeyers and wells where visible growth had occurred. These samples are diluted 10 times in liquid YES and spread on a YES agar plate. The same step was performed after 96 hours. After 48 more hours, a big and well-isolated colony was selected on each plate and streaked on an antibiotic containing YES media plate to eliminate potential contaminations and full revertant (suppressors that have lost the entire region encoding the flag and the resistance marker or a major part of it). These plates were placed at 37°C for 48 more hours and the surviving colonies were tested further as potential suppressor strains.

### **Total genomic DNA sequencing**

Total purification of genomic DNA was made on liquid YES expanded culture using Zymo research Kit for genomic DNA extraction (YeaStar™ Genomic DNA Kit) and following the adjunct protocol. After double-stranded DNA concentration measurement by QuBit, the DNA was fragmented by sonication in a bioruptor (Diagenode, Belgium) set on Low position (130 W) for 7 cycles of 30 seconds. A fraction of the sonicated DNA was charged on a 1 % agarose gel in order to check size of the obtained fragments. The resulting fragments were sequenced by Illumina sequencing at the Marie-Curie institute of Paris.

## Plasmid creation

All plasmids used in this study are based on the pREP3 vector. This replicative plasmid contains a leucine autotrophy marker *leu1* and a discrete start site for DNA replication *ars1*. It also contains an ampicillin resistance gene for selection in bacteria. The cloning site is preceded by a full length *nmt1* (no message in thiamine) promoter that is fully repressed after 18 hours on 15  $\mu$ M thiamine and fully expressed when no thiamine in the media [84].

The *Sla1* containing plasmid is based on this pREP3 vector, with insertion of the coding region, exon and intron included, but not the 5' or 3' UTR, at the cloning site. Insertion of the *slal* sequence was performed by restriction at the *SalI* and *BamHI* sites.

## Transformation

Transformation of yeast strains with plasmid vector pREP3 was performed using the lithium acetate procedure on exponentially growing culture [85]. Briefly, exponentially growing cells were resuspended in Lithium acetate buffer at high concentration ( $10^9$  cells/ml). Sonicated herring sperm DNA was added at the same time as the transformed plasmid in order to saturate nucleases and binding on the cell wall. Cells were rendered competent by the combined effect of the alkali cations contained in the lithium acetate, chemicals polyethylene glycol (PEG) and a heat shock at 42 °C. PEG promotes association of the transforming DNA with the surface of the cell while LiAc while the heat shock promote passage of DNA into the cell [86]. Then, cells were resuspended in EMM and spread on EMM agar plates. To avoid plasmid loss, the transformed strains are carrying the *leu1-32* allele which mean they are auxotroph for leucine. The pREP3 contains *leu1* to restore autotrophy for leucine in transformed strains.

## Northern blot

RNA from  $2.2 \times 10^8$  cells in exponential phase was extracted by the phenol-chloroform method [44] and was washed twice in 75% ethanol made in DEPC water at 4°C to get rid of the remaining salt/phenols. RNA extracts were air dried, then resuspended in DEPC water. The quality of the final RNA extracts (concentration and absence of contamination by salt and proteins) was assessed by nanodrop.

For visualization of tRNA, 8  $\mu$ g of this total RNA extract were denatured during 2 minutes at 95°C then charged on a 10 % poly-acrylamide urea gel, with equal volume of a sample buffer composed of formamide, EDTA and bromophenol. After migrating for 20 hours at 4°C and 110 V, the gel was semi-dry transferred on a Hybond-N (Amersham) membrane humidified with a solution made from Tris base, boric acid and EDTA. The transfer was made at 4°C and 400mA 6V for 45 minutes. The membrane is then washed in SSC 2x buffer solution. Finally, RNA is cross-linked to the membrane by a 30-seconds exposure to UV.

Radioactive labelling is performed by overnight incubation at 42 °C in hybridization buffer containing gamma-<sup>32</sup>P ATP (Perkin, 10mCi/mL) radiolabeled probe (**See Table S3 for probes sequence**). The membrane is then washed in SSC buffer 10% with 0.1% SDS at 42 °C and revealed by exposing a super RX-N film overnight at -80°C or using a Cyclone phosphorimager (2 to 6 hours).

For visualization of mRNA, more specifically in the case of *sla1* overexpression assessment, the same extraction method was used. Then 10.8 µg of the extract mixed with 10% of loading dye buffer were loaded on a 1% agarose gel stained with ethidium Bromide and containing formaldehyde. Migration lasted 1h30 at 110V. The gel was then washed in NaOH and in neutralization buffer, made of NaCl, Tris-HCl and DEPC treated water . The transfer was performed overnight by capillarity in Saline Sodium Sulfate (SSC) buffer to a Hybond-N membrane (Amercham). RNA is crosslinked to this membrane by 2 hours incubation at 80°C.

*Sla1* messenger RNA was probed using random primers labeling for long probes by incorporation of radioactive nucleotides. 25ng of PCR product, amplification of the sequence following intron of *sla1* until 3'UTR, were incubated 1h at 25°C with Klenow fragment, dinucleotides and alpha-<sup>32</sup>P dCTP. The product of the reaction was purified on a GE Healthcare column (ProbeQuant G-50 Micro Columns) then incubated with the membrane overnight at 65°C. The membrane was then washed in SSC buffer 10% with 0.1% SDS and revealed by exposing a super RX-N film 2 hours at -4°C or using a Cyclone phosphorimager (30 minutes at RT).

### **Chromatin Immunoprecipitation**

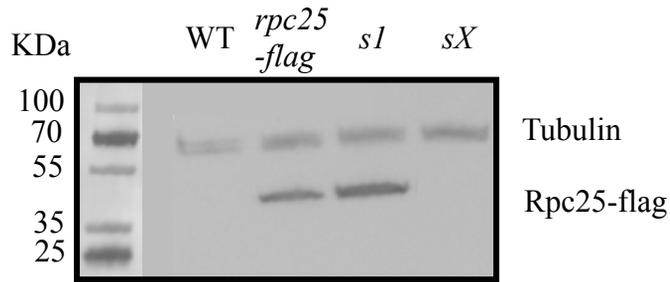
Chromatin immunoprecipitation was performed in order to establish if the addition of the flag on Rpc25 has an impact on the binding of the RNA polymerase III to its target genes. ChIP protocol starts with the crosslinking of proteins to their bound chromatin. The crosslinking was performed on 80 ml of a culture at OD 0.6 by the addition of 5 mL of formaldehyde 16%. The reaction was then stopped after 10 minutes by the addition of glycine in excess. Protease inhibitor was added before the chromatin extraction step. Afterward, yeast cell-wall were mechanically disrupted by 7 cycle on settings 6.5 of FastPREP-24 Classic Instrument (mpbio) with 1.5 mm zirconium beads and the extracts were separated from cell remnant by centrifugation. The chromatin was then disrupted by sonication in a biorupter (7 cycle on High position). The sonication product was homogenized than cell fragments were removed by centrifugation.

The immunoprecipitation was carried out using PAP antibodies coated on anti-mouse IgG Dynabeads (Invitrogen,Calrsbad,CA). Afterward, pronase (a mix of proteases) and RNase were added [63]. The product was purified on MSV Spin PCRapace column (Invisorb) following manufacturer instructions.

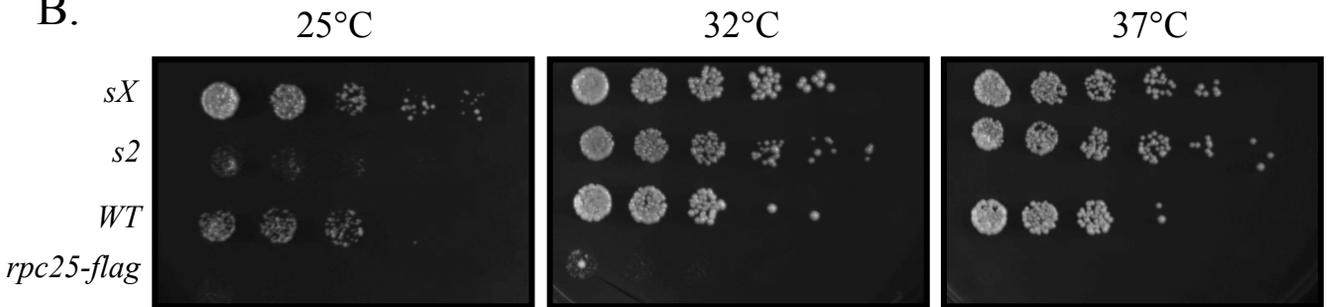
### **Real-time PCR**

Input and immunoprecipitated DNA samples were used as template in real-time PCR reactions in SYBR Green Mix (Applied Biosystems) on a 96 well Optical Reaction plates in an CFX96 Touch Real-Time PCR Detection System (Bio Rad) (**See Table S4 for probes used**). Relative quantification using a standard curve method was performed for each primer set than input DNA values were used to normalize ChIP. Enrichment levels were determined by comparison between total chromatin extracts and chromatin extracts after immunoprecipitation.

A.



B.



**Figure 8: Flag loss in *rpc25-flag* restore growth.** **A)** Cells of wild-type (WT), *rpc25-flag* strains and suppressors isolated from *rpc25-flag* were grown in liquid YES medium at 32 °C until exponential phase. After alkaline protein extraction, protein extracts were subjected to Western blot analysis using the appropriate antibodies (see Table S 2) and revealed by ECL. **B)** Spot dilution assay with serial dilutions of the wild type (WT) and the indicated mutant strains on YES medium. Plates were incubated for 72 hours at the indicated temperatures.

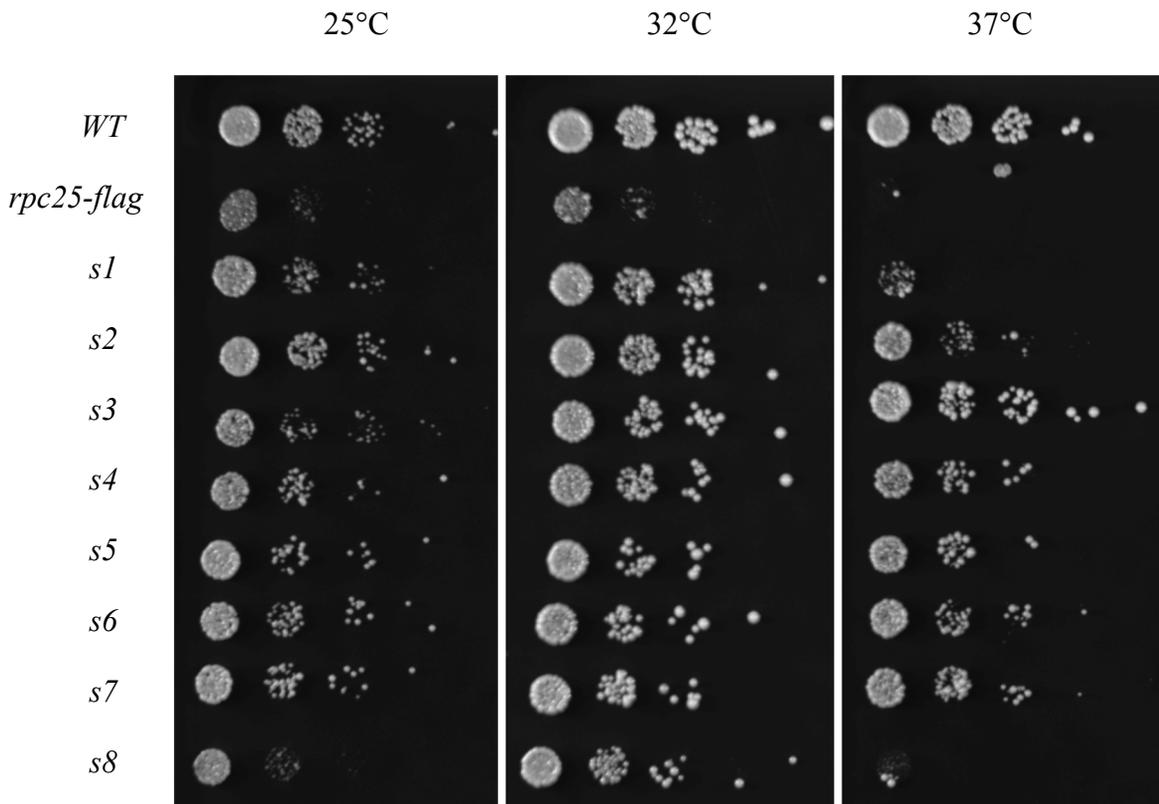
# Results

## Suppression of the *rpc25-flag* growth defect

### Isolation of suppressors of the *rpc25-flag* growth defect

In order to better understand the regulation of Pol III, we set up a screen to identify genes whose mutation suppresses the growth delay resulting from the *rpc25-flag* allele. The idea behind the screen is that the mutation of factors that negatively regulate Pol III could compensate for deficiencies in the *rpc25-flag* strain and thus restore normal or close to normal growth in the suppressor mutant strains. These suppressors are usually obtained by induced mutagenesis (ultraviolet, ethylmethanesulfonate, base analogs, ...) or by taking advantage of the natural mutation rate under selective pressure. In our case, as the ideal suppressor should have only one mutation differentiating it from the original strain, the natural mutation rate was a better option as it will result in a more restricted number of mutations in the suppressors. Suppressors obtained thus only resulted from mutational events normally arising in situations of growth at high temperature of a *S. pombe* population. High-throughput sequencing of the genome of the suppressors considerably facilitated the identification of the suppressive mutation, which previously required tedious genetic mapping. This methodology allowed eight independent suppressors to be isolated and sequenced in the course of this master thesis. The original *rpc25-flag* strain used as a basis for suppressor isolation was also sequenced for comparison, to exclude mutations that would have occurred between this strain and the *S. pombe* reference genome.

Many natural suppressors were isolated from growth at restrictive temperature. Some suppressors were rejected from further analysis due to the intragenic origin of their suppression. Indeed, for these mutants, a PCR designed to verify the integrity of the *rpc25-flag* allele was negative, suggesting that a rearrangement removed the flag tag, or modified the reading frame in the tag, which usually leads to premature STOP codons. In other rejected suppressors, as well as in some double mutants created by crossing of the *rpc25-flag* strain, the PCR for *rpc25-flag* was positive but Western blot probing the presence of the flag was negative and sequencing of one of these mutants (named *sX*) showed a deletion of 100 bp within the flag sequence. This deletion is located within the flag encoding sequence and is resulting in a 100 nucleotide band shift that is difficult to unambiguously resolve on an agarose gel but that makes the encoded product not recognizable by the antibody used for Western blot (**Figure 8 A**). Incidentally confirming that the *rpc25-flag* phenotype is genuinely caused by the flag, its loss in the *sX* strain fully suppresses the *rpc25-flag* growth defect (**figure 8 B**).



**Figure 9: Suppression of the *rpc25-flag* growth defect.** Spot dilution assay with serial dilutions of the wild type (WT) strain, the *rpc25-flag* original strain and its indicated suppressors strains (s1 to s8) on YES medium. Plates were incubated for 72 hours at the indicated temperatures.

Finally, eight potentially extra-genic suppressors were selected based on the integrity of the Rpc25-flag fusion. They will be referred to as *s1* to *s8*. In these suppressors, wild-type-like growth at restrictive temperature is restored as showed by drop dilution assay (**Figure 9**). Only *s1* and *s8* still demonstrated a slight growth defect. In the case of *s1* this suppressor was isolated from a previous spot dilution assay performed on *rpc25-flag* at 32°C instead of the isolation procedure described in the material and methods (at 37°C). Thus, the selective pressure resulting in the emergence of this strain was different, and likely less stringent, explaining how this mutant has been selected.

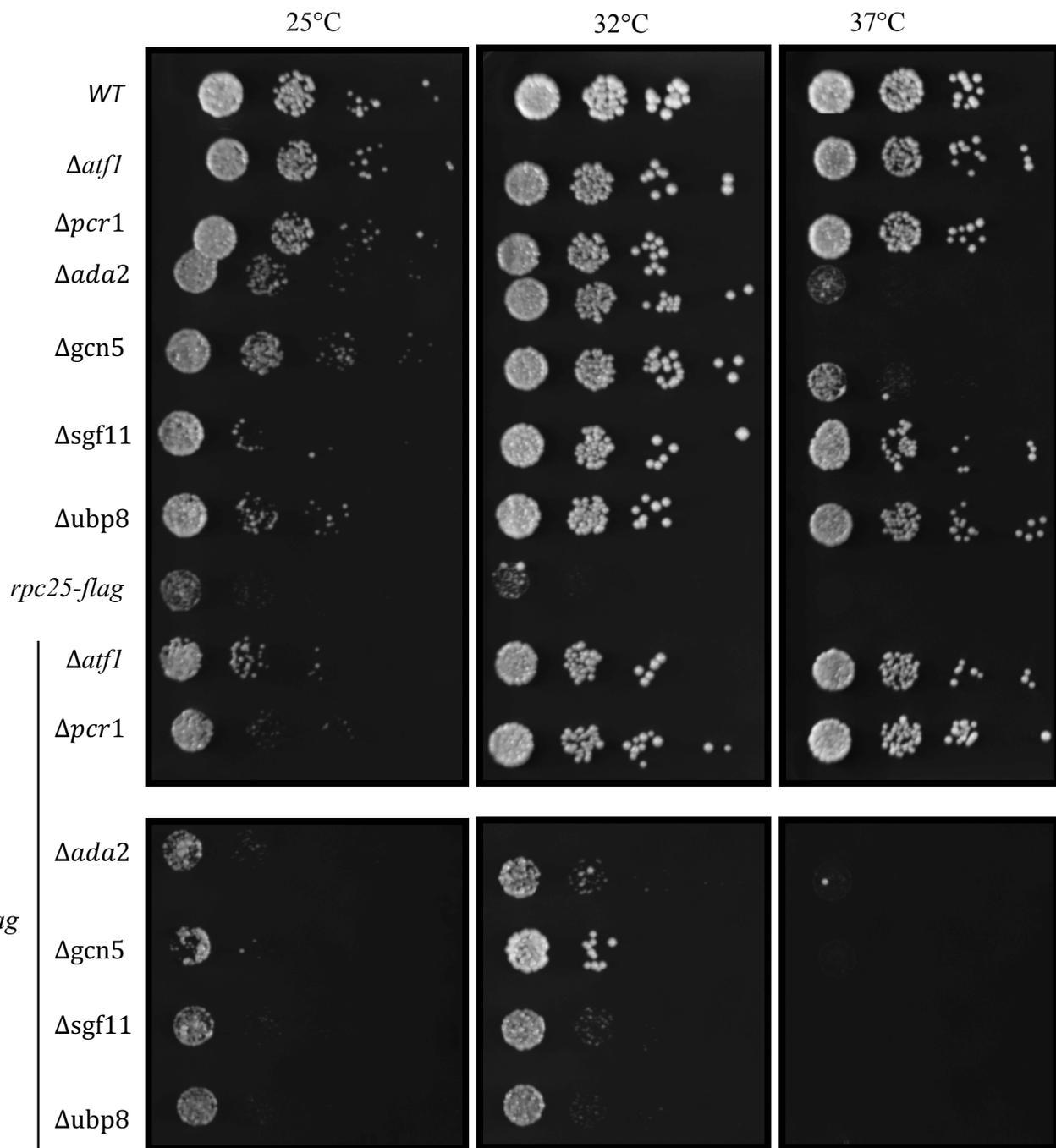
Next, precise localization of the mutations in the eight isolated suppressors was obtained by whole-genome sequencing. Detection of single nucleotide variations and of small indels was made possible by Illumina sequencing with a 60-fold genome coverage. Reads were mapped to the *S. pombe* reference genome and compared to this reference using varScan [87] in order to detect mutations. Mutations that were present in the majority of reads from one suppressors but absent in the majority of reads from *rpc25-flag* original strain were classified as potentially suppressive mutations.

**Table 1: Suppressor's mutations summary**

Mutation	Position and strand of the gene	Strain	Effect	Frequency
+T pcr1 ORF	I: 4252900 strand –	s1	Frameshift on 96,5% of the protein	17/22 reads
+G brf1 ORF	II:3058544 strand –	s1	Frameshift on 92,2% of the protein	13/25 reads
+A tra1 ORF	II:1901765 strand –	s2	Frameshift on 66,1% of the protein	29/35 reads
+A sgf73 ORF	III:2122889 strand-	s3	Frameshift on 13,5% of the protein	28/35 reads
C->A tms1 ORF	II:291803 strand –	s4	Silent	33/33 reads
G->A rps1901 3'UTR	III:3818953 strand+	s5	Unknown	21/34 reads
C->A tra1 ORF	II: 1902387 strand-	s6	Ser->Stop before 72% of the protein	23/29 reads
C->T mug133 ORF	I; 2264167 strand+	s6	Silent	33/38 reads
C->G mcs4 3'UTR	II:3559093 strand+	s7	Unknown	60/60 reads
T->A intergenic region	I:4706397	s8	Unknown	34/34 reads
A->G mss116 ORF	II:1429472 strand+	s8	Silent	76/76 reads

Retained mutations were present in at least 50% of the reads from the suppressors and 10% of the reads from the original *rpc25-flag* strain. Except from mutation within *brf1* and *rps1901*, all of these mutations were present in at least 75% of the reads. The potentially affected gene products were Pcr1, Brf1, Tra1 (two times), Sgf73, Tms1, Rps1901, Mug133, Mcs4 and Mss116. Mutations within *mcs4* and *rps1901* were located in the 3'UTR while the other were found within the open reading frames. Another mutation was detected though it was not localized in a gene but in an intergenic region lying between *klp3* and *pmo25*. Besides, two potentially suppressive mutations were detected in each of three suppressors. **Table 1** provides a summary of the potentially suppressive mutations, their localization and the type of mutation. If the mutation is inducing a frameshift or an early stop codon, the percentage of out of frame codons or untranslated codon within the protein was calculated.

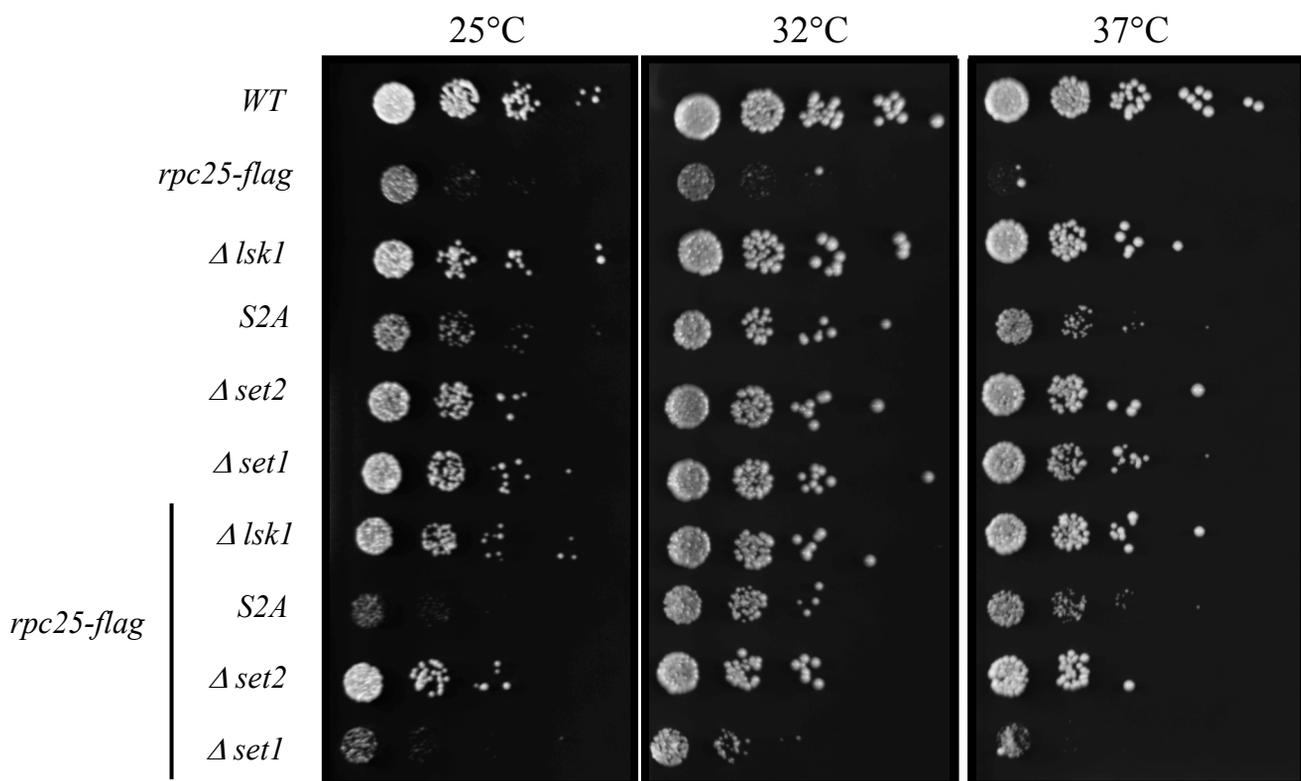
After isolation and sequencing, eleven punctual mutations were detected among eight suppressors strains. Each strain contains at least one mutation and a maximum of two mutations, which is a good results considering than the optimal number of mutation in our case, to ease further analysis, would be of one mutation per strain. These mutations lay in coding genes, 3'UTR and also intergenic region and consist in addition or replacement of a single nucleotide. Based on these information, further analyses were performed in order to more clearly define the pathways leading, when impacted, to a suppression of *rpc25-flag* growth defect.



**Figure 10 : Genetic interactions between *rpc25-flag* and SAGA subunits or recruitment factors.** Spot dilution assay with serial dilutions of the wild type (WT) strain, the *rpc25-flag* original strain as well as SAGA related protein deletion mutants and their corresponding doubles mutants. Plates of YES agar were incubated for 72 hours at the indicated temperatures. All strains were processed together and plated on the same plate, but the original picture was cut and rearranged for ease of viewing.

### Effects of mutations within SAGA-related proteins

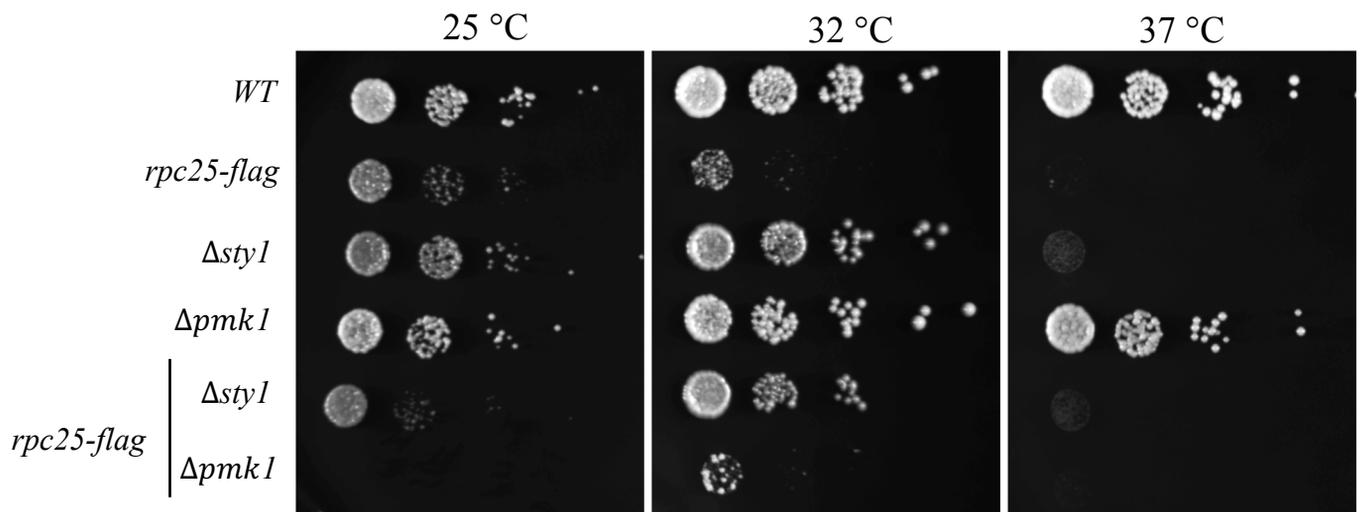
Among the mutations retained in the isolated suppressors, four are directly related to the SAGA complex (two located within *tral*, one within *pcr1* and one within *sgf73*). Among those, three are inducing a frameshift that impacts a significant part of the protein and one is causing a premature translational arrest. In order to both independently confirm the genetic interactions highlighted and further explore the connection between SAGA and the suppression of the *rpc25-flag* allele, double mutants were created by crossing the *rpc25-flag* strain with different mutants harboring full deletion of genes encoding SAGA-related proteins. The tested proteins are the SAGA subunits Gcn5, Sgf11, Ada2 and Ubp8. The SAGA-related proteins Atf1 and Pcr1, which form an heterodimeric transcription factor recruiting SAGA, were also tested. Deletion of *atf1* and of *pcr1* both result in a strong suppressive phenotype, including at 37°C, while none of the tested SAGA subunits induce such suppression, although *gcn5* deletion results in a slight suppression, hard to definitively assert based on this result alone (**Figure 10**). Altogether, those results indicate that deletions within subunits of the HAT and DUB SAGA modules are not sufficient to induce a suppressive effect, in contrast with the point mutations in *tral* and *sgf73* identified in the naturally occurring suppressors.



**Figure 11 : Suppression of *rpc25-flag* growth defect by S2A related mutants.** Spot dilution assay with serial dilutions of the wild type (WT) strain, the *rpc25-flag* original strain and other deletion mutants and their corresponding double mutants. Plates of YES agar were incubated for 72 hours at the indicated temperatures.

### Effects of mutations within proteins related to the CTD

In order to further investigate the relationship between the *rpc25-flag* growth defect and the phosphorylation of Pol II CTD, as pointed-out by results from Carlo Yague-Sanz (See **introduction and Figure 6**), other double mutants were created by crosses. The impact of the deletion of *lsk1*, *set2* and *set1* were tested by spot dilution assay (**Figure 11**). Deletion of *lsk1*, *set2* or *set1* alone has no detectable impact on growth, while *S2A* mutation on itself causes a decreased growth at 25°C and at 37 °C. The *S2A* mutation as well as the deletion of *set2* and *lsk1*, suppresses *rpc25-flag* growth defect without completely restoring a wild-type-like growth in the *rpc25-flag* background. The suppression by *S2A* is only effective at 32°C but this is explained by the fact that this mutation induces a cold-sensitive and heat-sensitive phenomenon, leading to a growth defect originating in Pol II transcription defect. These mutations are epistatic to *rpc25-flag*. Parallely, the deletion of *set1* does not suppress the *rpc25-flag* growth defect. This result points out that the suppressive effect obtained in the CTD S2A linked strains is specific to mutations affecting recruitment of protein by the phosphorylation of this serine and not to any mutation affecting the recruitment of proteins by modification of the CTD. Indeed, the CTD S2P is known to recruit Set2 but it is the CTD S5P that recruits Set1.

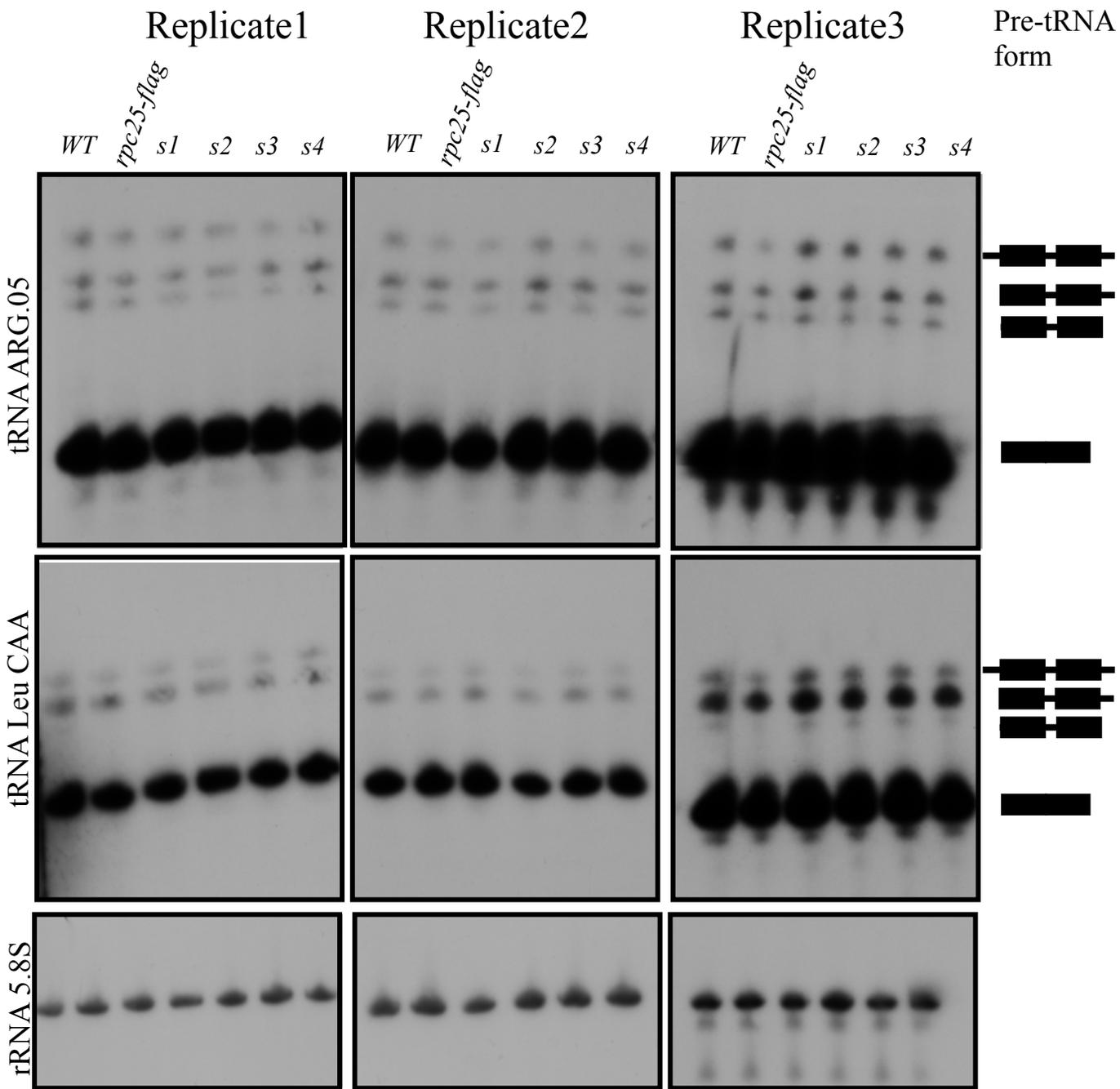


**Figure 12 : Suppression of *rpc25-flag* growth defect by MAPK depletion.** Spot dilution assay with serial dilutions of the wild type (WT) strain, the *rpc25-flag* strain as well as *pmk1* and *styl* strains and their corresponding double mutants. Plates of YES agar were incubated for 72 hours at the indicated temperatures.

### Effects of mutations within MAPK Sty1 and Pmk1

As the Sty1 MAP kinase directly phosphorylates both the Atf1 transcription factor and the Lsk1 CTD kinase, the capacity of the *styl* deletion to suppress the growth delay resulting from the Rpc25-flag fusion was tested. To better assert its specificity, another fission yeast MAPK (Pmk1) was also tested. There are only three identified MAPK in *S. pombe* and one of them (Spk1) causes penetrant sterility when deleted. Thus, only Pmk1 and Sty1 were tested.

After crossing between an *rpc25-flag* strain and a  $\Delta styl$  or a  $\Delta pmk1$  strains, the double mutants were tested by drop dilution assay. Deletion of *styl* induces a suppressive effect within the *rpc25-flag* strain at 32°C but is not effective at 25°C, for unexplained reasons, and not effective at 37°C most probably because *styl* deletion on itself induces a heat sensitive phenotype, as Sty1 is involved in the cellular response to heat [88]. In contrast, *pmk1* deletion has no suppressive effect and presents no phenotype in the tested conditions (**Figure 12**). This leads to the conclusion that not every MAPK mutation results in a suppressive effect that is specific for *styl*.



**Figure 13 : *rpc25-flag* suppressors tRNA profile.** Northern blot analyses of pre-tRNA content in WT, *rpc25-flag* and four suppressors. Strains were grown in liquid YES medium at 32 °C until exponential phase. RNA extracted by phenol-chloroform was analysed by northern blot analysis with A) a probe against SPBTRNAARG.05. B) a probe against LeuCAA C) a probe against 5.S rRNA. Each replicate correspond to one membrane sequentially incubated with the different antibodies.

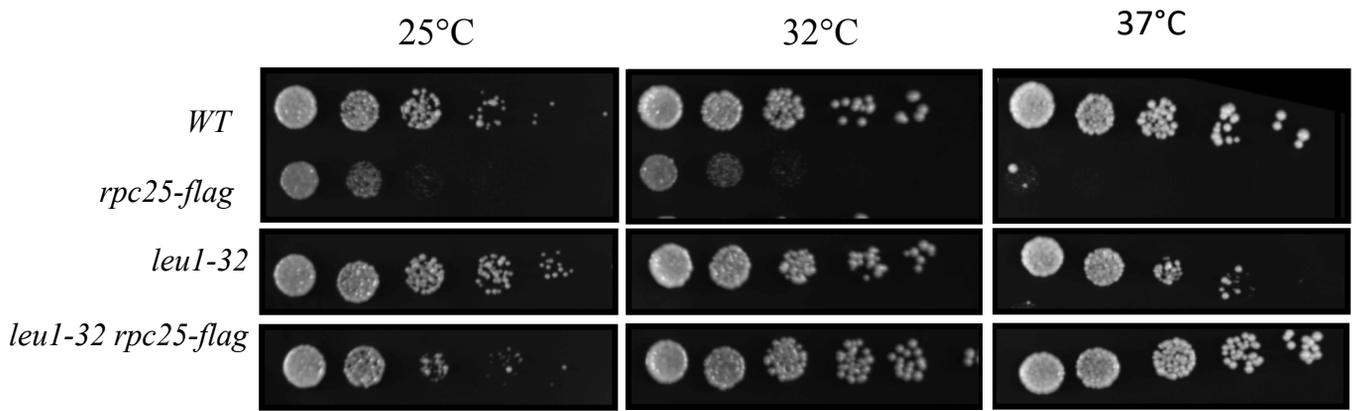
## Link between restoration of growth and increase in pre-tRNA level

*Rpc25-flag* known phenotypes include reduced growth and pre-tRNA content. The isolated suppressor strains were selected because the mutations they contain improve growth of this strain. We then asked if the improved growth in the suppressors come along with an increase in pre-tRNA level, which would link the rescue of growth to the restoration of normal tRNA levels. After total RNA extraction and migration, two tRNAs and the 5.8S rRNA were detected with radioactive probes and exposition on R-X films. The rRNA 5.8s is shown has a loading control, as this rRNA is transcribed by Pol I and is not expected to be affected by mutations impacting Pol II or Pol III. Also, it is present in large amount within the cell compared with pre-tRNA, and thus easily detected.

Three independent biological replicates were performed to determine the level of pre-tRNA within the suppressors. The loading control shows that a similar amount of total RNA has been charged for each strain, although small punctual differences can be perceived. Despite similar methodology and solution in each replicates, the third one is of higher quality, with best resolution and each pre-tRNA forms easily recognizable. This third replicate is also the only one in which a clear reduction in pre-tRNA level can be seen in the *rpc25-flag* strain compared with the wild-type, with no observable difference in the loading control between these two conditions. In this third replicate, the level of pre-tRNA is globally higher within the suppressors compared with the *rpc25-flag* base strain, reaching levels similar to those of the wild-type strain, although slight increases or decreases can be observed depending on the strain and pre-tRNA form (**Figure 13**).

In contrast, no restoration of pre-tRNA level can be perceived in the *rpc25-flag* suppressors for the two other replicates. However, the difference of pre-tRNA level between *WT* and *rpc25-flag*, well established in previous results from the lab, is not obvious. This is probably due to the fact that these replicates are of lower quality, as pre-tRNA are highly unstable and only present in low quantity within the cell, their extraction and detection can be quite tricky. Similar effects can be seen for the two pre-tRNA tested, tRNA Leucine with complementarity to CAA codon and tRNA Arginine encoded by SPBTRNAARG.05 but tRNA Arginine detection gives more clear and sharp results, which could be explained by a higher stability, a better extraction of this pre-tRNA or a higher affinity of the used probe.

A definitive conclusion would be premature based only on these three replicates, as their quality is very different. The third replicate seems more reliable as it matches earlier results, where a deficiency in pre-tRNA level within the *rpc25-flag* strain was observed (**Figure 6**) and both the signal intensity and the resolution is high. For this replicate, the conclusion would be that restoration of pre-tRNA level within the suppressors correlates, and might be causal, in the suppression of the *rpc25-flag* growth defect. However, further high-quality replicates will be necessary to establish this conclusion.

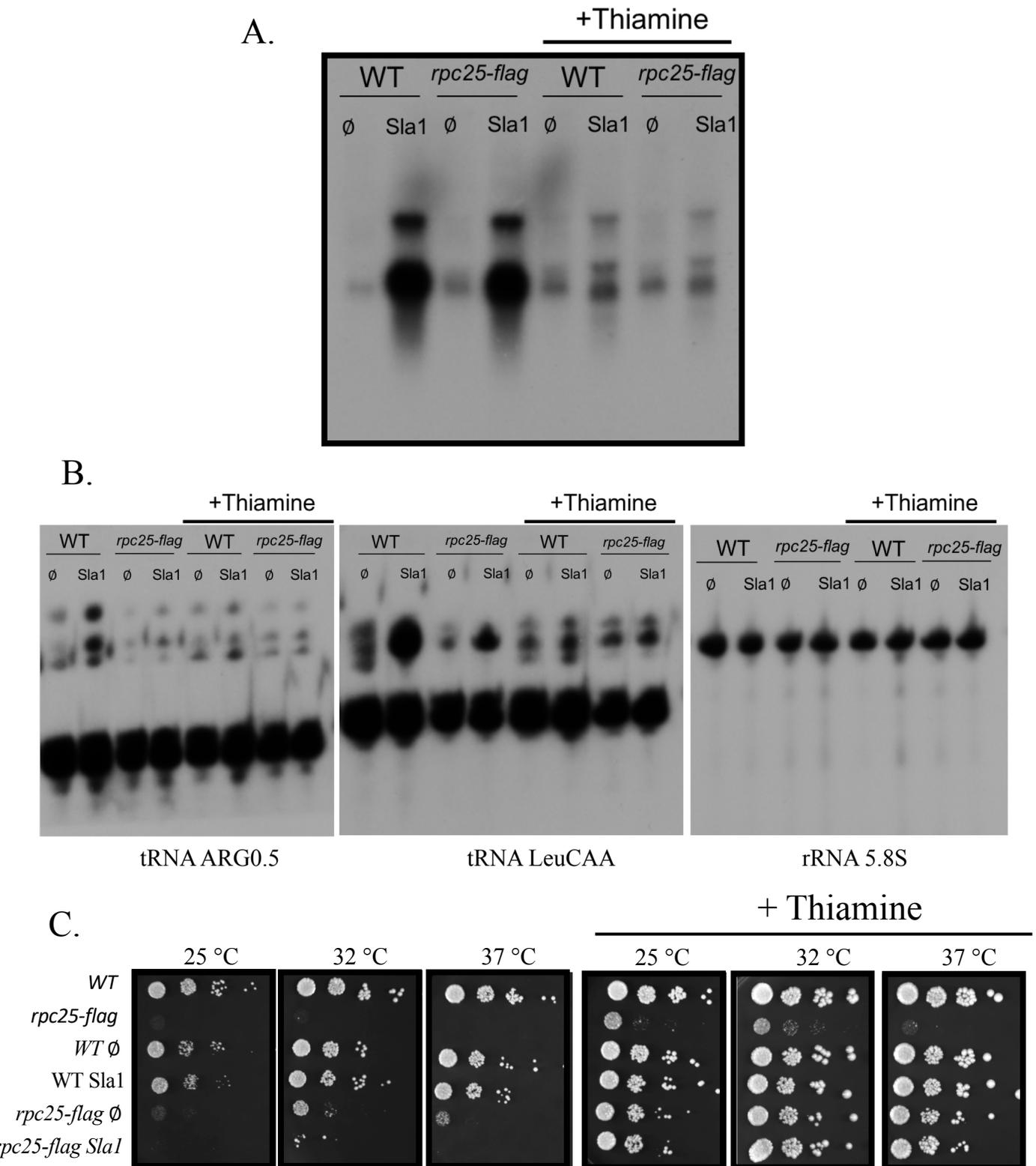


**Figure 14 : Suppression of the *rpc25-flag* growth defect by mutation within *leu1*.** Spot dilution assay with serial dilutions of the wild type (WT) strain, the *rpc25-flag* original strain and its indicated suppressors strains on YES medium. Plates were incubated for 72 hours at the indicated temperatures. All strains were processed together and plated on the same plate, but the original picture was cut and rearranged for ease of viewing.

### Effect of mutation within *leu1*

We next created an *rpc25-flag leu1-32* strain in order to select for the pREP3 vector (that contains a *leu+* allele complementing the leucine auxotrophy of the *leu1-32* allele) was tested in a preliminary suppressor assay. Unexpectedly, the mutation have a suppressive effect on *rpc25-flag* growth defect phenotype at 25°C, 32°C and 37°C. This *leu1-32* allele has no impact on *WT* growth (**Figure 14**). Further test realized with this strain indicates that this suppression is dependent on the presence of thiamine within the media, but these tests were realized after transformation with a *leu1* containing plasmid and thus this result is not entirely transposable to the *leu1-32 rpc25-flag* strain (**Figure 15 C**).

When complementing in condition without thiamine, the suppression is much less obvious, which seems to indicate that the presence of thiamine is critical for the suppression. However, when complementing in condition with thiamine, EMM+ thiamine or YES (that contains thiamine), the suppressive effect persist. It then seems like thiamine has an effect on the growth of *rpc25-flag* and that this effect can synergize with the suppressive effect of *leu1-32*, even when complementing this allele (**Figure 15 C**).



**Figure 15 : Sla1 overexpression increases the level of pre-tRNA and negatively impact growth in an *rpc25-flag* strain.** **A)** Northern blot analyses of pre-tRNA content in WT or *rpc25-flag* transformed with empty ( $\emptyset$ ) or NMT1 *sla1* containing (Sla1) plasmids. Strains were grown in liquid EMM, with or without Thiamine, at 32 °C until exponential phase. RNA extracted by phenol-chloroform was analysed by northern blot analysis with a probe against *sla1*. **B)** Northern blot analyses of pre-tRNA content in WT or *rpc25-flag* transformed with empty ( $\emptyset$ ) or NMT1 *sla1* containing (Sla1) plasmids. Strains were grown in liquid EMM, with or without thiamine, at 32 °C until exponential phase. RNA extracted was analysed by northern blot with probes against tRNA ARG0.5, tRNA LeuCAA and rRNA 5.8S sequentially applied on one membrane. **C)** Spot dilution assay with serial dilutions of the wild type (WT), *rpc25-flag* and the transformed mutants strains, all *leu1-32*, at the indicated temperatures

# Sla1 overexpression does not restore growth of *rpc25-flag*

## Sla1 overexpression increases pre-tRNA level

To test the correlation between restoration of pre-tRNA level and of growth level in the *rpc25-flag* strain, a strain where the pre-tRNA levels are artificially increased without direct mutation of Pol III or of its associated transcription factors was created. To do so, we transfected *S. pombe* cells with *sla1*-containing replicative plasmids as described [48]. Indeed, as explained in the introduction, Sla1 is a tRNA chaperone known to stabilize pre-tRNAs. To check that *sla1* is effectively overexpressed when strains are transformed with the *NMTsla1* plasmid in an inductive media, a northern blot probing the *sla1* messenger RNA was performed. Three bands are detected for each condition. Compared with cultures transformed with an empty plasmid, the global amount of signal is higher in cultures transformed with the *sla1*-containing plasmid. For these, as expected, growth in induction media (without thiamine) further increases the signal. In the same conditions, there is no detectable difference in *sla1* mRNA level between the wild-type strain and the *rpc25-flag* strain. The two bands whose size does not correspond to the 1185 nt expected for Sla1 mRNA probably result from the retention of Sla1 mRNA by the extremely abundant rRNA 25S (3400 nt) and rRNA 18S (1800 nt) (**Figure 15A**).

To check if overexpression of Sla1 efficiently increases the pre-tRNA levels in the transformed strains, northern blot were performed using the same probes as for quantification of pre-tRNA within the suppressors. As expected, the overexpression of Sla1 causes an increase in pre-tRNA level within the cell, in both the wild type or in the *rpc25-flag* strain though this increase is far larger in the wild type. As the addition of thiamine within the media is not sufficient to completely inhibit the NMT1 promoter, responsible for the expression of *sla1* on the plasmid, a slight increase in pre-tRNA level can also be observed within the strains transformed with the *sla1*-containing plasmid in repressive media. Visualization of rRNA 5.8S, transcribed by Pol I and thus not expected to be impacted by a Pol III mutation, serves as a loading control and indicates that a similar amount of total RNA was loaded in each well (**Figure 15B**).

## Impact of *sla1* overexpression on *rpc25-flag* growth

To determine whether or not the overexpression of *sla1* in the *rpc25-flag* strain is able to restore growth, a drop dilution assay was performed on both a wild-type strain and a *rpc25-flag* strain transformed with an empty or a *sla1*-containing plasmid. The four transformed cultures were tested in media inducing the overexpression of *sla1* (EMM) or repressing its NMT1 promoter (EMM + thiamine).

When allowing overexpression of *sla1* (EMM media without thiamine), the wild-type strain growth is not robustly impacted, regardless of the kind of plasmid transformed or of the temperature. In contrast, the *rpc25-flag* strain is systematically growing less than its appropriate WT controls. Specifically, Sla1 overexpression has clearly no suppressive effect on *rpc25-flag* growth. In fact, compared with the condition where an empty plasmid is transfected, Sla1 overexpression appears to aggravate the growth defect phenotype (**figure 15 C, left panels**).

As an additional control, we also assessed the growth of these strains in conditions where *sla1* is not overexpressed (EMM + thiamine). However, to our surprise, the presence of thiamine itself has a partial suppressive effect on the growth of *rpc25-flag*. Moreover, this suppression has at least an additional (perhaps synergic) effect with the *leu1-32* allele (that we saw also

have suppressive effects– see **Figure 14**) even if *leu1-32* has been complemented with the *S. cerevisiae leu1* gene within the transfected plasmids (**figure 15 C, right panels**). Because of those complex suppressive effects, the conditions with thiamine are probably not the best controls for this experiment. Nevertheless, it appears clearly from these assays that Sla1 overexpression *per se* is not suppressive of the *rpc25-flag* allele.

## TORC1 activation in *rpc25-flag*

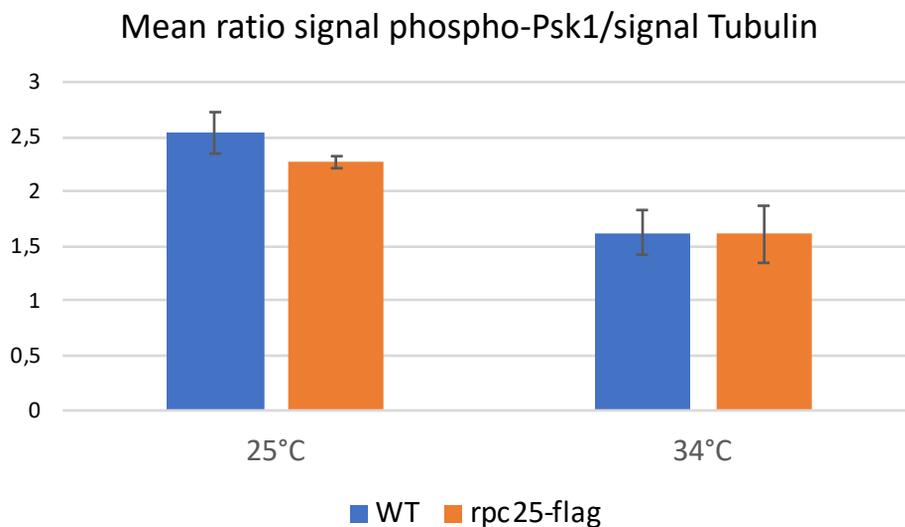
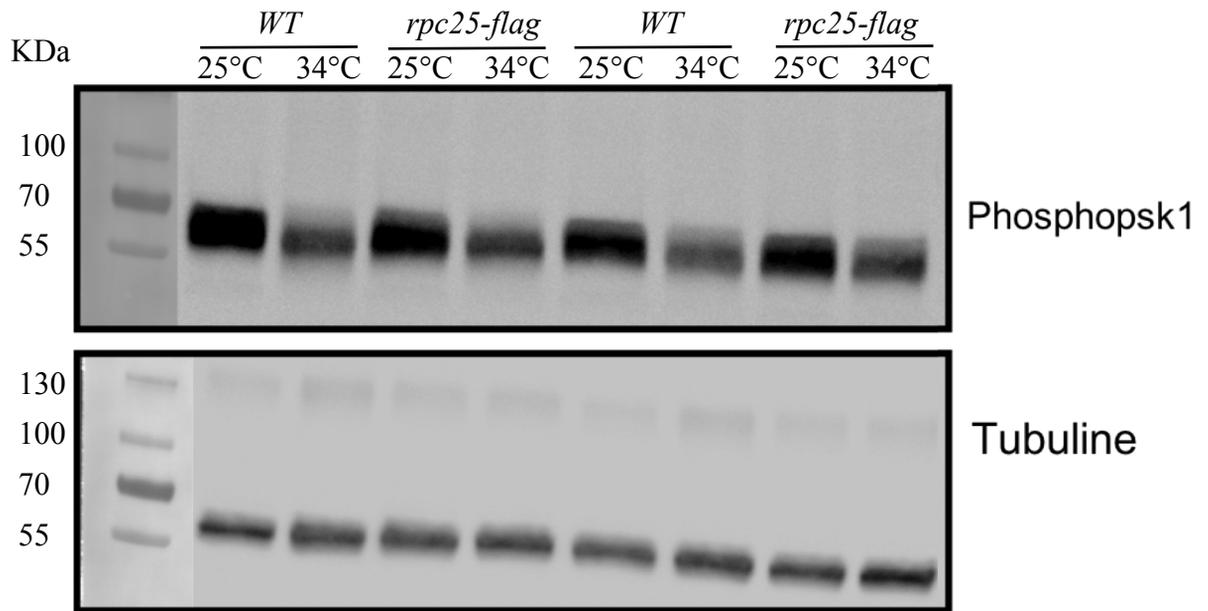
### Antibodies against human phospho-p70 S6 kinase recognize the homolog Psk1

A recently published article reports a direct relationship between pre-tRNA level in *S. pombe* cells and the activation of TORC1, a complex responsible for regulation of many pathways, including sexual differentiation [48]. As the *rpc25-flag* mutant conveniently presents a decreased level of pre-tRNA, a variation of TORC1 activation in our mutant would confirm these results and potentially make of *rpc25-flag* a useful tool to study TORC1. If the activity of TORC1 is impacted in the *rpc25-flag* strain, the restoration of TORC1 normal activity in the suppressors would also be an interesting issue to test. To assess physiological differences between the *rpc25-flag* strain and a *WT* strain at the level of TORC1 activation, the level of phosphorylation of Psk1, a well-characterized TORC1 substrates, was tested.

First, the quantification of the level of phosphorylation of Psk1 was assessed through the detection of the band shift resulting from Psk1-Myc phosphorylation using an antibody against the Myc tag. The phosphorylation/unphosphorylation of Psk1 induces a slight difference in migration, resulting in two bands, the lower, fast migrating corresponding to the unphosphorylated form and the higher, slow migrating, to the phosphorylated form. However, as the band shift is subtle, it is challenging to differentiate the two forms and to independently quantify them (data not shown).

Therefore, in a second attempt to assess a potential difference in Psk1 phosphorylation, we used a mouse monoclonal antibody directly recognize the phosphorylated form of Psk1. Initially, the antibody was selected for the recognition of a phosphorylated peptide of the human phospho-p70 S6 kinase. Although the two orthologs are very dissimilar, with an identity of 49 % at the protein level, the antibody against the human protein also recognizes the fission yeast phospho-Psk1 protein with high specificity (no other detectable band) [48]. Confirming the specificity of the antibody for Psk1, the addition of thirteen repetitions of the Myc sequence causes a difference of about 40 kDa in migration distance of the entity recognized by the antibody, entity that we can therefore unambiguously design as Psk1. This is much larger than the size of the Myc repetitions (15.6kDa, thirteen times 1.2kDa). This fact is easily explainable by the fact that Myc does not contain any positively charged residue but do contains aspartic and glutamic acid that will slow its migration on the gel [89] (**Supplementary Figure 1 A**).

In addition, the specificity of this antibody for the phosphorylated form of Psk1 was tested by shifting of a wild-type culture from minimal growth media (EMM + N) to minimal growth media without nitrogen (EMM - N) for 30 minutes prior to protein extraction. This shift is known to induce a strong decrease in Psk1 phosphorylation [90]. Looking at the resulting blot, the amount of Phospho-Psk1 detected in nitrogen depleted media is indeed greatly reduced relatively to the level of Tubulin (**Supplementary Figure 1 B**).

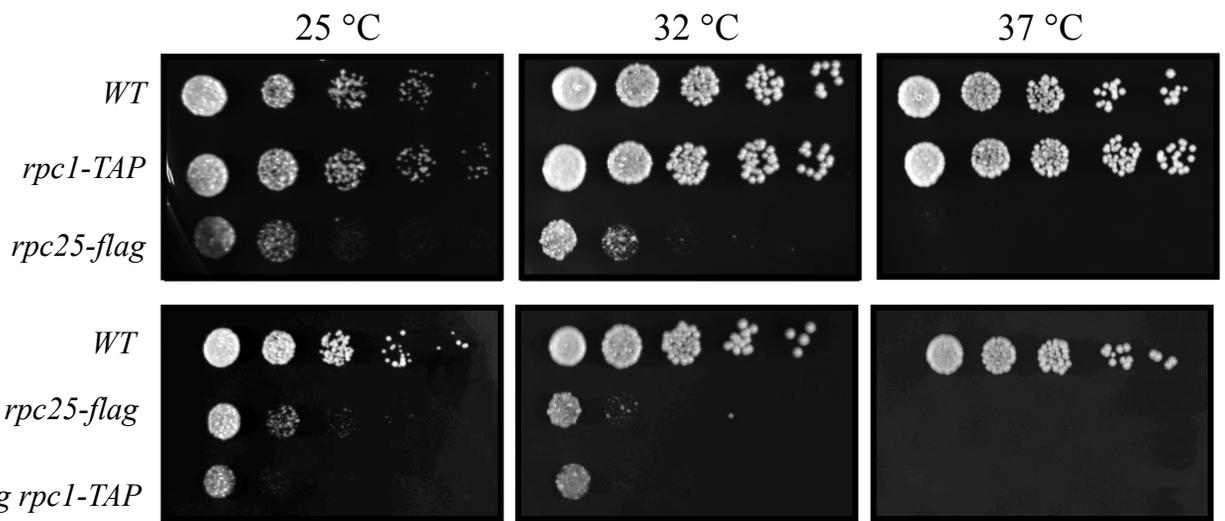


**Figure 16 : Phosphorylation of Psk1 is unaffected by the *rpc25-flag* mutation.** Cells of *rpc25-flag* and *psk1-13Myc* strains were grown in liquid medium at 32 °C until mid-exponential phase (DO= 0.4), then shifted to 25°C or 34°C for 4 hours. After TCA extraction, protein extracts were subjected to Western blot analysis using the appropriate antibodies (see Table 2) and revealed by ECL (upper panel). Signal quantification of this revelation is made by Imagequant analysis of the 3 replicates with standard error (lower panel).

### Phospho-Psk1 level of *rpc25-flag* is similar to wild-type

After validation of the phospho-Psk1, western blot were performed on protein extract from *WT* and *rpc25-flag* culture grown at 25°C or 34°C. Phospho-Psk1 was then specifically detected using the anti-phospho-Psk1 antibody and normalized on Tubulin. The membrane was stripped between incubation with anti-phospho-Psk1 and anti-Tubulin to get rid of the antibody and the chemiluminescence it would have induce.

For each strain and condition the ratio of the phospho-Psk1 level on Tubulin level was calculated. Standard error for the three replicates performed in each condition were comprised between 3 and 16 % of this ratio. At 25°C the value of the ratio in *rpc25-flag* was of 89% of the *WT* ratio while at 34 °C the ratio in *rpc25-flag* was of 99% of the same value in the *WT* strain. Making the same comparison about temperature, the ratio at 34°C in the mutant and in the wild-type represent respectively 64% and 71%. This apparent bigger difference arising from different temperatures than from different strains is confirmed by statistical analysis. Two-ways ANOVA analysis indicates that the only significant factor is the temperature, which is highly significant while strain and interaction between strain and temperature have no significant impact on the level of Psk1 phosphorylation normalized on Tubulin (**Figure 16**). These results indicate that the *rpc25-flag* mutation have few or no effect on TORC1 activity.



**Figure 17 : Growth phenotype of the *rpc1-TAP rpc25-flag* double mutants.** Spot dilution assay with serial dilutions of the wild type (WT) and the indicated mutants strains on YES medium. Plates were incubated for 72 hours at the indicated temperatures.

## Characterization of the *rpc25-flag* strain

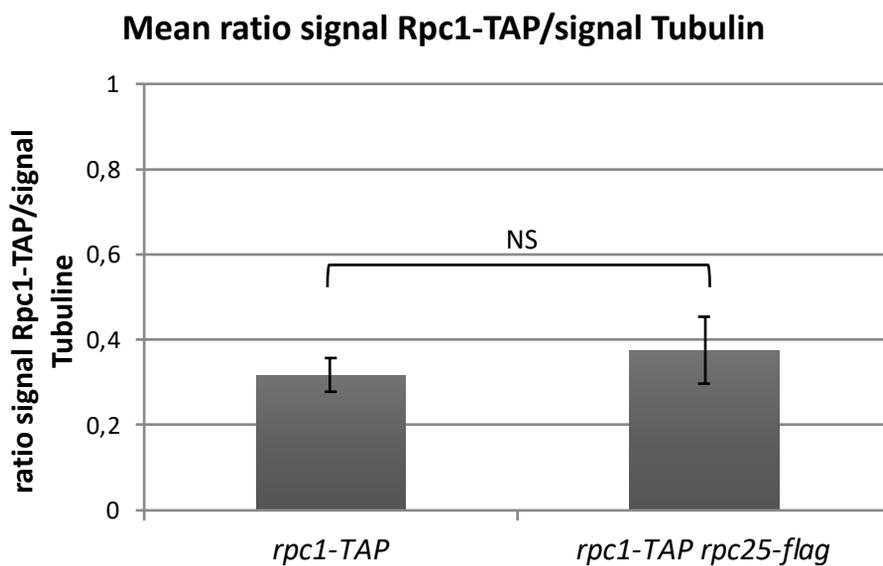
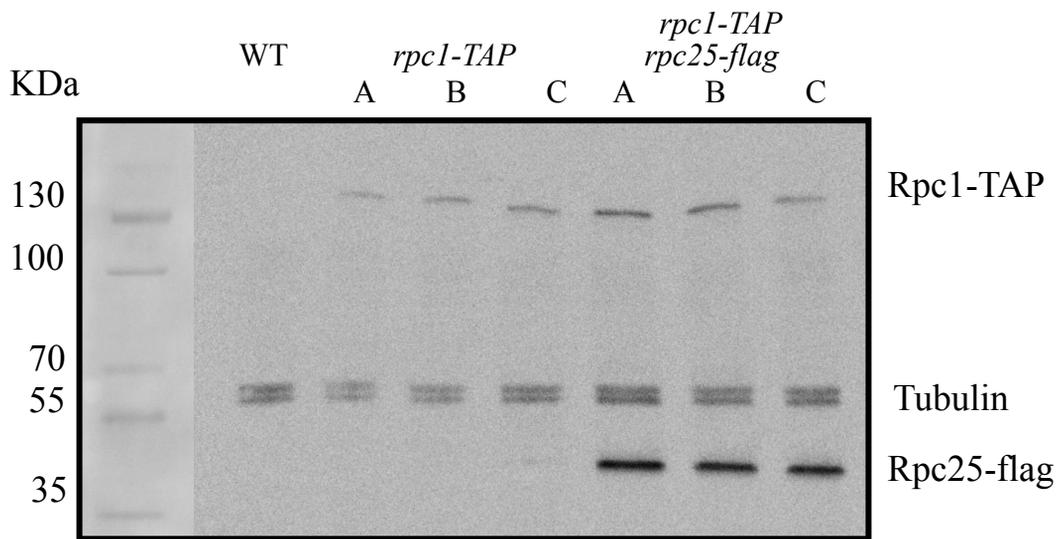
### The level of the catalytic subunit of Pol III, Rpc1, remains unchanged in the *rpc25-flag* strain

With the aim of obtaining a better characterization of the *rpc25-flag* mutant, the tagged *rpc1-TAP* was used. The addition of the TAP on Rpc1, Pol III largest and catalytic subunit has not been linked to any phenotype. This additional tag allows us to assess the fate of Rpc1 in the *rpc25-flag* strain, in term of global amount within the cell as well as in term of binding to the DNA.

Candidates double mutants harboring both the *rpc25-flag* allele and the *rpc1-TAP* allele were obtained by crosses, as described. Two validated strains, one of mating type h<sup>+</sup> and one of mating type h<sup>-</sup> have been selected, they will be referred as *rpc1-TAP rpc25-flag*. These strains showed the same kind of growth defect as *rpc25-flag* single mutant, as mutation of *rpc1* by a TAP fusion is not suppressing, nor worsening the phenotype (**Figure 17**).

Quantification was performed by comparing the single *rpc1-TAP* mutant to the *rpc1-TAP rpc25-flag* double mutant in order to assess if the presence of a Rpc25-flag fusion has an impact on the relative amount of Rpc1 and, by extension, on the average number of Pol III complexes in the cell. Bradford quantification of protein concentration following TCA extraction gave concentration ranging from 3µg/µl to 4.88 µg/µl. From each extract, 10µg of proteins were separated by SDS-PAGE. The normalized level of Rpc1-TAP on Tubulin was obtained from three biological replicates for which cell cultures and protein extraction were performed independently while migration, transfer and western blotting were performed on the same gel and membrane. After normalization on total Tubulin, no significant difference between the mean level of Rpc1-TAP was detected between the control and the *rpc25-flag* strains. The mean ratio for *rpc1-TAP rpc25-flag* replicates is 118% of the same value for *rpc1-TAP* with standard error between the three replicates of 7.2% of the *rpc1-TAP* ratio and 12% of the *rpc1-TAP rpc25-flag* ratio. Student test indicates a p value superior to the significant threshold, and the difference between the mean ratio between the two strains is thus considered non-significant (**Figure 18**).

The conclusion of quantification of Rpc1-TAP in the *rpc25-flag* mutant is thus that there is no significant difference detectable in the level of the Rpc25 subunit in the *rpc25-flag* mutant compared to wild type, at 32°C in YES. Also, the presence of a TAP tag on Rpc1 has no phenotypical consequences in the tested condition.



**Figure 18 : Rpc1-TAP relative amount in *rpc25-flag* and wild type strains.** Cells of wild-type (WT), *rpc1-TAP* and *rpc1-TAP rpc25-flag* strains were grown in liquid YES medium at 32 °C until exponential phase. After TCA extraction, 10 µg of protein extracts were subjected to Western blot analysis using the appropriate antibodies and revealed by ECL. Signal quantification is made by Imagequant analysis of the 3 replicates (lower panel). Mean ± Sd values of three independent measurements are shown. Not significant  $P > 0.05$  (Student's t-test).

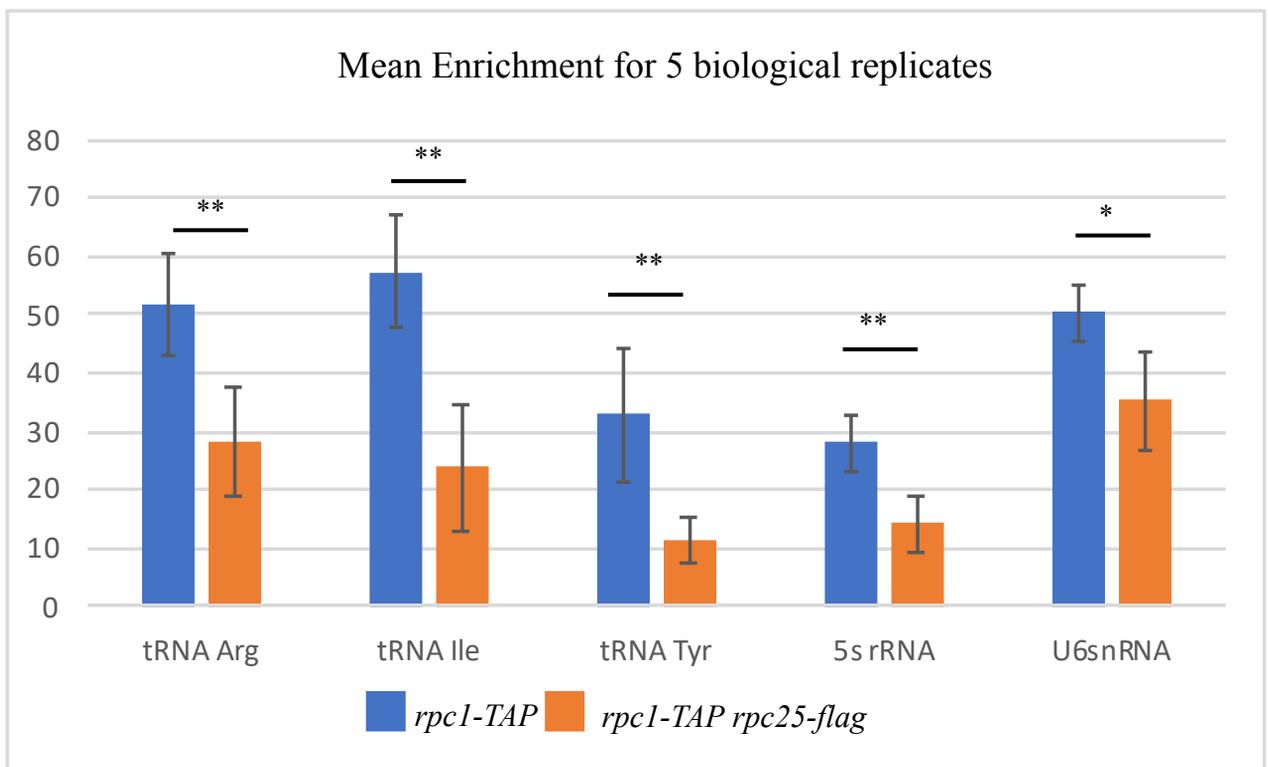
### **The occupancy of Rpc1 is decreased at class III genes in the *rpc25-flag* strain**

We used the already created *rpc1-TAP rpc25-flag* strain to analyze the occupancy of Pol III by Chromatin Immunoprecipitation (ChIP).

The occupancy of Pol III on the *act1* gene is used as an internal negative control, as Pol III is not supposed to bind this Pol II transcribed gene [18]. Indeed, the binding of Rpc1 to *act1* is very low, as expected (**Supplementary Figure 2A and B**). Mean enrichment (IP/T ratio) for the Pol III transcribed genes in an *rpc1-TAP* background was on average 33.37% on five different genes, five biological replicates and two technical replicates by biological replicates. Validating further the specificity of the ChIP assay, the quantification performed on a wild-type strain or on a *rpc25-flag* strain harboring an untagged Rpc1, resulted in very low level of occupancy defining the background. The *act1* negative control is based on the same five biological replicates while only two technical replicates were made for the non-TAP-tagged strains negative control (**Supplementary Figure 2B**).

The results indicate a significantly lower occupancy of Rpc1 on class III genes in the *rpc25-flag* background, compared to wild type. This statement is valid for the five Pol III-transcribed genes tested, one Arginine tDNA, one Isoleucine tDNA, one Tyrosine tDNA, the 5S rDNA and the U6 snDNA and based on the enrichment from five independent biological replicates, with two technical replicates for each one (**Figure 19**). For these loci, a significant (p value < 5%) decrease of about two-folds in enrichment level in the *rpc25-flag* strain was observed.

We therefore conclude that the slow growth and low pre-tRNA level observed when a Rpc25-flag fusion is expressed is correlated with a decreased occupancy of Rpc1 on chromatin at class III genes loci while the total level of Rpc1 in the cell is unaffected.



**Figure 19 : *rpc25-flag* impacts binding of Pol III to its transcribed genes** *rpc1-TAP* and *rpc1-TAP rpc25-flag* strains were grown in liquid YES medium at 32 °C until exponential phase. After crosslinking and chromatin extraction and sonication, DNA bound to Rpc1-TAP was co-immunoprecipitate, and measurement of the relative amount of four genes was performed by Real-time PCR (see corresponding probes **Table 2**). The average enrichments (percentage IP/total) for five biological replicates with error bar corresponding to confidence interval are shown. Significance indications based on student t-test with alpha equal 5%.

# Discussion

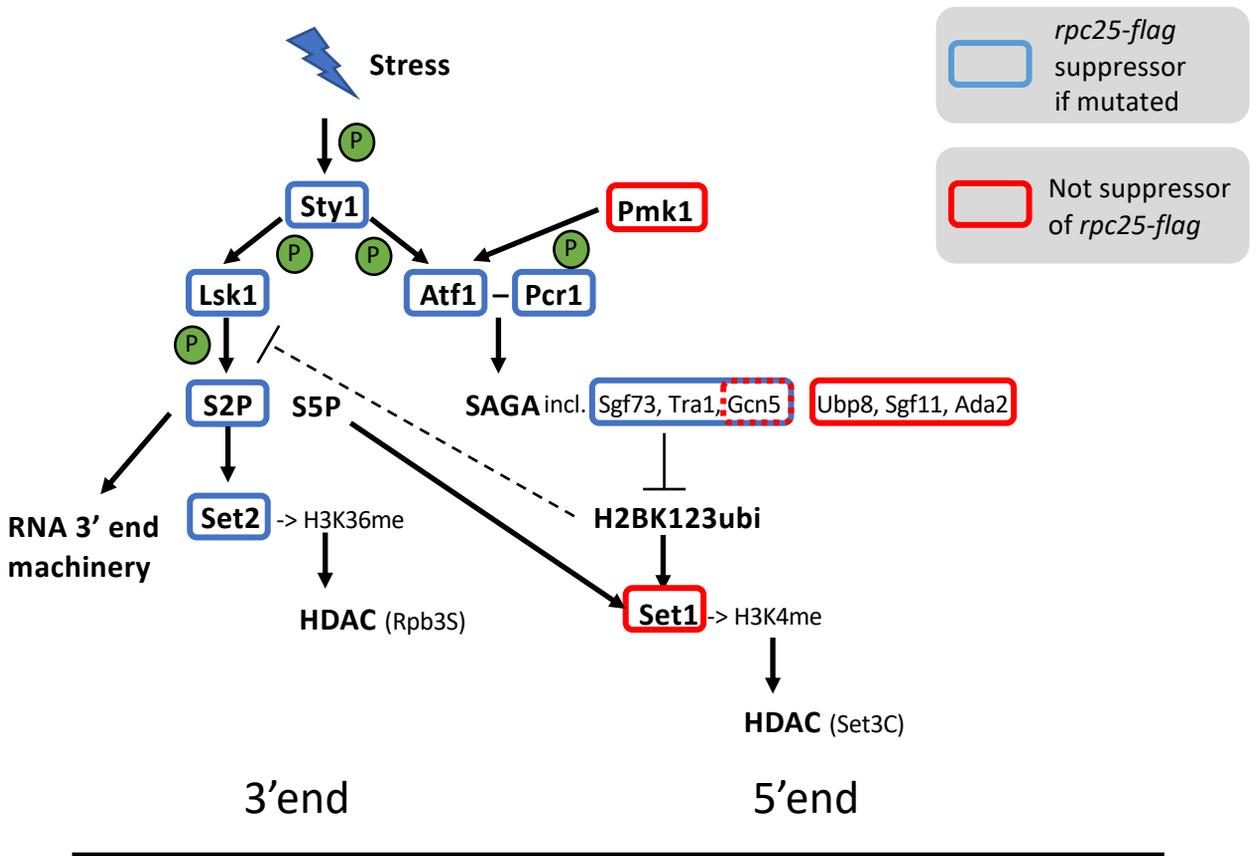
## Natural suppressors mutations implicate new pathways for Pol III regulation

### **Are suppressive effects of *tra1* and *sgf73* independent from SAGA?**

Four of the potentially suppressive mutations identified in the suppressors are linked to the Spt-Ada-Gcn5 acetyltransferase (SAGA) complex. Either they directly concern a subunit of the complex (Sgf73 and Tra1) or they affect a transcription factor implicated in its recruitment (Pcr1) [73, 79]. This strongly suggests a link between SAGA and Pol III activity. Looking closer at the SAGA subunits causing suppression of *rpc25-flag* phenotype, it seems that the deubiquitinase module is specifically impacted [73]. Indeed, Sgf73 is part of the DUB module and is required for its activity and Tra1, among other things, is required for the assembly and activity of this module [77,91].

We thus decided to test the effects of inactivation of other components of the DUB module (namely Ubp8 and Sgf11) on the *rpc25-flag* growth defect. Importantly, the deletion of different members of the DUB module cannot be considered fully equivalent to the deletion of Sgf73 as they have different impact on expression of Pol II-transcribed genes, indicating distinct roles within the complex [77]. However, as they impact the DUB activity of SAGA, their deletions were predicted to be suppressive and yet were not. Thus, Sgf73 and Tra1 may have another common function outside of the DUB module, which would explain their specific suppressive effect. Remarkably, the structure of SAGA should be deeply affected anyway. Sgf73 is playing the role of an anchor between the DUB module and the core of the complex [75]. Thus, deletion of this subunit would result in the detachment of the complete module and in a strong variation in the global structure of the SAGA complex. Same consequences are expected for Tra1 that represents almost half the size of the complex and plays a major role in regulation and recruitment of the other subunits.

Yet, the suppression could still be unrelated to SAGA as Sgf73 and Tra1 are independently implicated in chromatin remodeling. Tra1 is part of the ASTRA (ASsembly of Tel, Rvb and Atm-like kinase) complex, which is not well characterized but participates in the remodeling of chromatin [75,92,93]. Sgf73 is also implicated in the RNA-induced transcriptional silencing complex (RITS). RITS triggers post-transcriptional silencing and also chromatin modifications leading to transcriptional silencing [75]. Thus both Tra1 and Sgf73 are implicated in chromatin remodeling processes independently from their belonging to the SAGA complex.



**Figure 20 : Functional relationship between *Rpc25-flag* suppressors.** Deletion or frame-shift for the genes coding for the proteins squared in blue are demonstrated *rpc25-flag* suppressors while deletion of those in red are demonstrated as not suppressive. Relation only demonstrated in *S. cerevisiae* is represented in a dashed line [67,70,79,80,99,124,127].

### **Recruitment of Set2 by S2P has a negative impact on Pol III transcription**

While either an unphosphorylatable CTD S2 (*S2A*), the deletion of *lsk1* or the deletion of *set2* suppresses the growth delay of the *rpc25-flag* strain the deletion of *set1* does not. This indicates some specificity in the suppressive effect arising from disruption of the S2P-dependent recruitment of Set2 (**Figure 20**).

While the loss of Set2 impacts H3K36 methylation, along transcribed genes, Set1 loss in contrast impacts H3K4 methylation near the TSS (Transcription Start Site) of these genes [96, 97]. Both modifications results in the recruitment of a different histones deacetylase (HDACs), namely Rpd3S for H3K36me and Set3C for H3K4me [98]. A possibility exists that the specific loss of the Rpd3S HDAC creates an hyperacetylated template at tDNA loci, helping the deficient initiation of Pol III in the *rpc25-flag* strain. Indeed, as Pol II transcription at class III genes is antisense to Pol III [63], Set 2 would be acting nearest to the TSS of tDNA where it would be promoting a more closed chromatin state through deacetylation.

### **Sty1 deletion is suppressive through its activity on Lsk1**

The Atf1 transcription factor and the Lsk1 CTD kinase share two interesting properties. Their absence suppresses the *rpc25-flag* dependent growth defect and they are both regulated by the Sty1 MAP kinase [63,67,80]. Moreover, one of the isolated suppressors contains a mutation that affects a protein uphill of the phosphorelay leading to Sty1 activation, Mcs4. These facts support a role of the classical MAPK Sty1 in the regulation of Pol III. Strengthening this possibility, the absence of Sty1 but not of another MAPK, Pmk1, suppresses the *rpc25-flag* defect (**Figure 12**). However, as Pmk1 also phosphorylates Atf1 [99,100], we propose that a specific Sty1 -> Atf1-Pcr1/Lsk1 relation is in operation in the context of Pol III.

### **Other biological functions that could have a link with Pol III transcription**

Some detected mutations lay in genes with unknown function or with a function that has never been directly linked with Pol III or Pol II. One of them can be indirectly linked with Pol II transcription: the mutation detected in the *mcs4* 3'UTR. Mutation within this part of the gene could affect transcriptional and post-transcriptional processing. Mcs4 activates a phosphorelay system in response to stress, resulting in the activating phosphorylation of Sty1 [104]. As *sty1* deletion also results in a suppression, the *mcs4* mutation could act on the same MAPK pathway discussed in the previous section.

The mutation detected in *s4* is localized in *tms1* and changes a glycine encoding codon (GGC) into a valine (GUC). Both residues side chains are apolar and non-aromatic. Thus the mutation is not expected to heavily perturb the protein function, unless this glycine residue is critical, which remains a possibility. The non-essential Tms1 protein is poorly described, its function of hexitol dehydrogenase only inferred from homology [105]. Intriguingly, a single report indicates that it binds human p53 expressed in fission yeast. There is no p53 homolog in our model organism, but when the human p53 is expressed in *S. pombe*, it causes a growth arrest, highlighting the conservation of the cell cycle regulation across the eukaryotic species [106]. This arrest can be rescued by overexpression of *tms1* because Tms1 binds p53 C-terminal domain, which inhibits its activity [105]. However, these data give little insight on Tms1

function in *S. pombe* and are difficult to connect with the rescue of the Pol III defect in *rpc25-flag*.

One of the detected mutations is both localized in a gene hard to relate to Pol III or Pol II and in an untranslated region (UTR). It lays in *rps1901*. Rps1901 is a structural component of the ribosome, homolog to ribosomal protein 19 in human and very conserved, although its deletion is viable because a paralog gene, *rps1902*, remains present to ensure its function in ribosome biogenesis [107]. Although, it is hard to determine how it could be related to Pol III transcription regulation.

Finally, in the *s8* suppressor, the only detected mutation is not localized in the coding part of a gene or even in its untranslated region. It cannot be related to an effect, even potential, on any known gene as this region is unannotated. However, the possibility remains that a previously undetected gene is lying at this localization, although it seems unlikely in a model organism with a relatively small genome that have been extensively studied. The punctual mutation could also impacts one of the bordering genes (*klp3* and *pno25*) or even a more distant gene. The bordering genes themselves cannot be directly related to Pol III as they respectively encode a kinesin-like protein and a protein implicated in cell polarity maintenance [108,109].

It is possible that another mutation is present in each of these suppressors but could not be detected as the analysis method is blind to large genome rearrangements, for example a gene duplication. Moreover, the deletion or addition of fragment longer than a handful of nucleotide would not have been noticed either.

### **How can a mutation deleting an essential gene be tolerated?**

Mutations discussed until now are all located in non-essential gene [77,88,105,107,110,111]. In contrast, one of the mutations of the first candidate suppressor genes, *brf1*, affects an essential gene, as Brf1 is necessary for proper activity of the TFIIB complex [107]. It is therefore less clear how the frameshift is tolerated within this strain.

However, we noticed that only about 50% of the reads support that frameshift, which is lower than for any other mutations. A good explanation for this contradiction would be that *brf1* has been duplicated in this suppressor. Thus, one of the paralog genes could produce an inactive protein while the other remains non-mutated and active, resulting in a viable strain. Moreover, a second mutation was detected in that suppressor strain, impairing *pcr1* and present in a majority of the reads. It is thus possible, or even likely given the link of *pcr1* with the other suppressive mutations, that the observed suppression is caused by this second mutation. Furthermore, the suppressive character of *pcr1* deletion has been confirmed in a double mutant. Because of this, *brf1* mutation is not likely to be suppressive and *pcr1* mutation within this mutant is the best explanation for its increased growth.

### How is *leu1-32* mutation suppressive?

Leu1 is implicated in the synthesis of leucine and the levels of amino-acids are impacting different pathway within the cell, for example TORC1, in particular when they are implicated in protein synthesis [112,113]. This could explain why *leu1-32* is a suppressive mutation. Still, discordant elements concern suppression by *leu1-32*. First, suppression of the growth defect phenotype by *leu1-32* is independent from the presence of leucine in the medium. This suppression is also independent from leucine autotrophy in a thiamine containing media, as demonstrated by the fact that complementation with *leu1* on the plasmid does not cause the return of the growth defect on EMM+ thiamine.

As leucine content within the cell does not seem to be the impacting element in *leu1* mutation, remaining hypothesis explaining an effect of *leu1-32* on Pol III transcription is that the enzyme encoded by *leu1*, 3-isopropylmalate dehydrogenase, has a role in another pathway than leucine synthesis that could explain its suppressive effect, or imbalance some metabolic branches targeting Pol III. This remains to be investigated. The complex interaction of thiamine with the phenotype observed stands on the same line and has not been investigated further here. Thiamine is a cofactor in the pentose phosphate pathway, citric acid cycle, glycolysis and branched amino-acid catabolic pathway. Fission yeast is able to synthesize thiamine, which means the thiamine content within the media is not limiting for growth. This molecule is also an important gene regulator, with regulation of more than 60 genes in *S. cerevisiae*. Its main targets are genes implicated in thiamine biosynthesis, sexual agglutination and zygote formation [114-117]. This molecule affects a wide range of cellular pathways and thus, its potential effect on Pol III transcription is hard to assess.

### ***Sla1* overexpression is toxic for *rpc25-flag***

At the beginning of this project, it was already known that pre-tRNA level within the *rpc25-flag* strain is reduced, while it is increased in a *S2A* mutant and that a *S2A rpc25-flag* double mutant restored both growth and pre-tRNA to a WT level (**Figure 6, Results from C. Yague-Sanz**). To assess whether the compensation of pre-tRNA level is causal in the suppression of the growth defect of *rpc25-flag*, we thought of artificially modify the level of pre-tRNA independently of transcription. To do so, *sla1* is overexpressed in *rpc25-flag*. *Sla1* is a pre-tRNA chaperone whose overexpression increases the level of pre-tRNA [48,50].

When comparing *rpc25-flag leu1-32* transformed with an empty or a *sla1* containing plasmid in inductive media (EMM) a slight decrease in growth can be seen in the strain overexpressing *sla1*. Thus, the overexpression of *sla1* does not improve growth of the *rpc25-flag* strain and even seems to worsen the growth defect. This negative effect of *sla1* overexpression on growth has already be seen in a temperature sensitive *tor2* mutant. However, in this other mutant, TORC1 activity was heavily impacted, while detection of phosphorylated Psk1 in *rpc25-flag* showed no difference in the activation of TORC1 [48].

We propose that the increased level of pre-tRNA detected when overexpressing *sla1* may not functional or available for the cell, precisely because they are associated with *Sla1*. In fact, *Sla1* impacts on pre-tRNA levels is due to its ability to bind pre-tRNA and thus to protect them from degradation [48]. However, this protection could also prevent them from maturation. Also,

binding to Sla1 could prevent pre-tRNA from binding to other regulatory elements, precluding any positive effects in the *rpc25-flag* background.

To sum up, the goal of this experiment was to test if a transcription-independent stabilization of pre-tRNA levels would be sufficient to suppress the growth delay of the *rpc25-flag* strain. Unexpected and complex genetic interactions between *leu1-32* and *rpc25-flag*, together with effect of thiamine on growth make the interpretation of the results difficult. In any case, the stabilization of pre-tRNA that is observed upon overexpression of *sla1* aggravates the growth defect. A possibility to explain this result is that Sla1 traps the already low level of pre-tRNAs.

## **Mutation of *rpc25* has no detectable impact on TORC1 function**

Mutation of Pol III is expected to impact TORC1 activity as pre-tRNA level is known to play a role in the regulation of this complex. The phosphorylation level of Psk1 in the wild-type and in the *rpc25-flag* strain were compared and no significant strain dependent difference on the phosphorylation level of Psk1 could be detected (**Figure 16**). This absence of strain-dependent difference in the phosphorylation of Psk1 is unexpected because *rpc25-flag* shows a major common phenotype (the reduction in pre-tRNA levels) with mutant strains analyzed in the study from Otsubo et al., 2018, and that these strains demonstrate a marked difference in TORC1 activity. Besides, another subunit of Pol III, Rpc34, was identified in this other study as causing an inactivation of TORC1. On top of that, Rpc34 has been linked with Rpc25 by structural studies [118,119].

It was also interesting to check if a difference in TORC1 activity arises when the temperature is higher, as *rpc25-flag* growth defect is more marked at this temperature. However, despite the fact that the difference in phosphorylation level depending on the temperature is significant, for both strains, no significant difference arises from the interaction of strain and temperature. This slight difference dependent on the temperature is expected, given that previous studies also showed a slight difference in phosphorylation level in wild-type strains depending on the temperature [48]. As this difference is of the same importance in the wild-type than in the *rpc25-flag* strain our conclusion is that there is no difference in the level of activation of TORC1 in our mutant, at least in the tested conditions.

## **What is the impact of *rpc25-flag* mutation?**

### **Information resulting from our study**

Several hypotheses can be formulated to explain the origin of *rpc25-flag* growth defect and reduction in pre-tRNA level. They all implicate a global decrease of Pol III activity resulting in a lower transcription rate but could affect different step of transcription, the stability of the complex or of its independent subunits. Western blot and CHIP of Rpc1-TAP in the context of a *rpc25-flag* mutant have provided precious information on the reason of the defect allowing us to refute some of these hypotheses.

First information, coming from quantification of the amount of Rpc1-TAP within the cell showed that it is not affected by the Rpc25-flag fusion. This allows us to reject one hypothesis, as it indicates that the strong decrease in pre-tRNA level observed when Rpc25 is fused to the

flag does not come along or result from a decrease in the abundance of Rpc1, the Pol III catalytic subunit. Afterwards, ChIP of tagged Pol III was ought to determine if the flagged Pol III is defective at the initiation or elongation (and less likely termination) steps.

The ChIP experiment indicated that the occupancy of Rpc1 is strongly decreased and therefore the *rpc25-flag* phenotype is unlikely to result from a lower elongation rate or from a lower termination rate, as those defects would rather cause an increased occupancy of Pol III at its transcribed genes.

The hypotheses we favor to explain the *rpc25-flag* Pol III defect are as follow: first, the assembly or the stability of the complex could be reduced in the mutant, without affecting the stability of the independent subunits, or at least of Rpc1. Secondly, the initiation could be less efficient in the mutant. Finally, we do not exclude a weaker binding of the complex to the DNA, which would result in a higher dissociation rate during elongation.

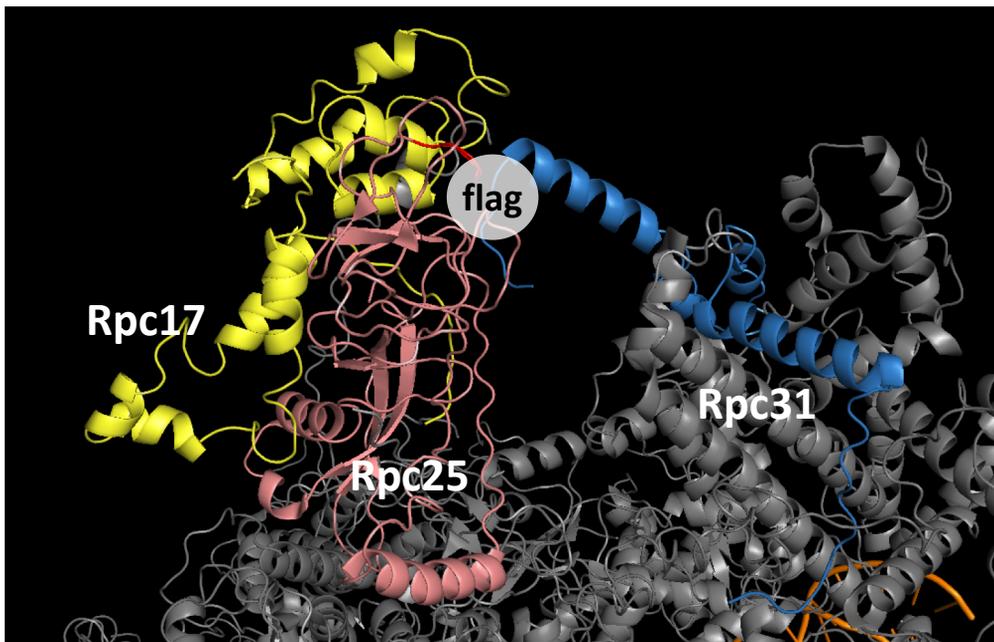
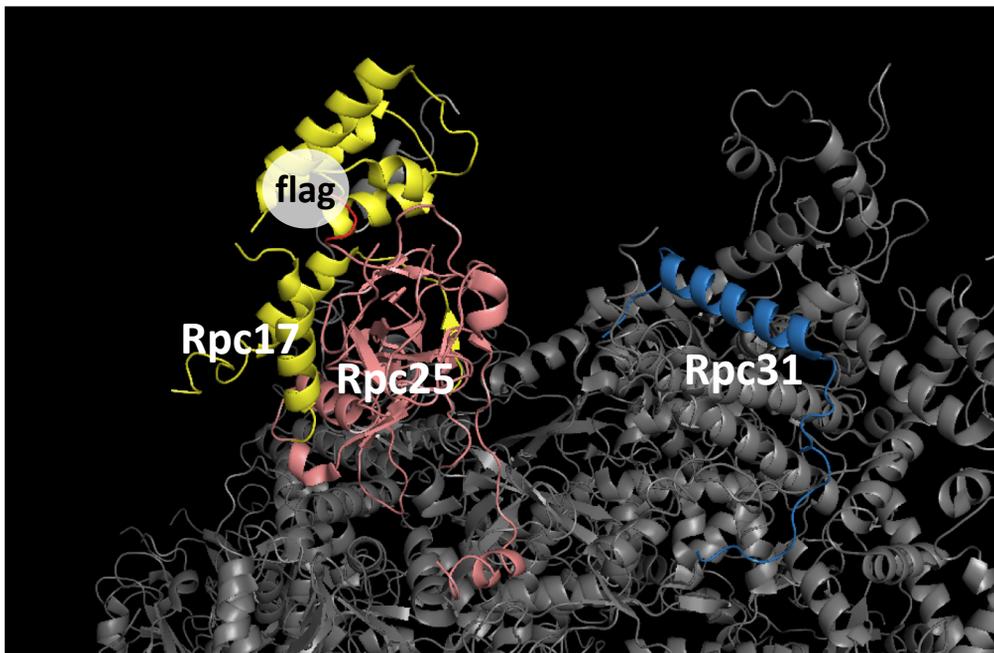
### **In the context of literature**

Results from independent studies can help us determine which hypothesis is the most likely. These studies have been performed in *S. cerevisiae* but, as polymerase complexes are very conserved, the gathered information are likely to be valid also in fission yeast. They rely on cryo-EM structural studies of Pol III at different step of the transcription initiation process [118,119] and on functional study of punctual mutants for *rpc25* [26].

This functional study showed that phenotype caused by mutation within *rpc25* does not originated from a defect in elongation or termination of transcription but from a defect in initiation, assembly or stability of the complex, as we have also proposed. The cryo-EM structures of apo-Pol III and Pol III in complex with TFIIB with or without binding to the DNA showed that this subunit could be capital for the conformation change that comes with the initiation step of the transcription. However, they were performed in wild-type strains and thus the effect of the flag on the structure can only be inferred.

These cryo-EM structures consider transcription initiation (from a structural point of view) as a three steps process following binding of TFIIB by Pol III. The polymerase complex can adopt two major conformations, depending on the position of the C82-C34-C31 heterotrimer. These two conformations are named “close” and “open”, they represent the first and the last step of initiation. These two states are separated by a transition step, where the inner clamp open, allowing DNA to enter the active site. Then, this clamp closes around the DNA, leading to the “open” conformation. This last conformation correspond to a state where DNA is bound inside of the polymerase groove and where DNA at the promoter is opened, forming the so-called transcription bubble.

This C82-C34-C31 heterotrimer, differentiating the “close” state from the “open” state, is composed of three subunits that are not conserved between polymerases. These subunits are thought to be resulting from the permanent recruitment of transcription factors. In this case, they are homologous to the Pol II general transcription factor TFIIE [13]. This heterotrimer, and in particular C34, has been shown to be important for the formation of the Open Complex, by stabilizing the transcription bubble, and is also essential for the recruitment of TFIIB. Thus, mutations within subunits of this sub-complex affect transcription initiation [120]. During initiation of transcription, a rearrangement occurs in this complex, leading to a tighter binding of the polymerase to the transcription bubble [118].



**Figure 21 : Rpc25 conformational change upon initiation.** Expected position of the flag at the C-terminal end of Rpc25 in the Apo pol III open complex (upper panel, pdb 6EU2) and the Pol III open pre-initiation complex (lower panel, pdb 6EU0). Pictures are made in PyMol based on structures from Abascal-Palacios et al, 2018. Rpc17, Rpc25 and Rpc31 are respectively colored in yellow, pink and blue [121] .

In cryo-EM structures of the polymerase complex, the stalk (the structure composed of Rpc25 and Rpc17) of Pol III seems to be implicated in the global conformational change driven by the C82-C34-C31. A precise region of Rpc25 appeared to be responsible for binding to the heterotrimer C82-C34-C31 when domain rearrangement leading to the formation of the PIC occurs (**Figure 21**). Moreover, binding region is located at the C-terminal domain of Rpc25, which is also the spot where the flag was added. This C-terminal part, in previous structural studies of apo-Pol III, was located outside of the complex. But after conformational change it rotates to the inner part of the complex in close contact with Rpc31. In this newly uncovered conformation a flag addition on Rpc25 C-terminal domain would have dramatic consequences on the initiation process as it would prevent the binding of the stalk to the heterotrimer. Such interference would have a strong impact on transcription initiation by Pol III. These structural deductions are coherent with previous functional studies [26] and arise independently from two independent studies published this year [118,119], which made them highly reliable.

The combination of quantification and ChIP of Rpc1-TAP gave us insights into the reason behind growth defect and reduction in the amount of pre-tRNA within the mutant. Still, this is not sufficient to specifically point out the origin of the transcription defect, as a lower binding to the DNA could result from several defects. However, some previous experiments link Rpc25 to initiation, and more specifically to the C34-C31-C82 subcomplex implication in the open complex formation [26,118–120].

## Restoration of pre-tRNA level within the suppressors

In the first known suppressor, the double mutant *S2A rpc25-flag* was restoring both wild-type growth and level of pre-tRNA. We thus asked if both effects occur in the isolated suppressors. At this stage, results from the three Northern blot replicates do not correlate and we cannot draw strong conclusion about the restoration of pre-tRNA levels in the suppressors.

If suppression of the growth defect does not go along with restoration of pre-tRNA level, this would mean that both phenotypes are not always correlated and leave two interesting hypotheses. First hypothesis, a decrease in pre-tRNA level does not cause the growth defect in the *rpc25-flag* strain. Pol III does not only synthesize pre-tRNA. It is also responsible for the synthesis of other non-coding RNA, which depletion could cause the growth defect, while decrease in pre-tRNA would be tolerated by the cell. This hypothesis would be supported by the fact that the active form, mature tRNA, show no significant decrease in quantity in *rpc25-flag* (**Figure 6 and 13**).

The other hypothesis that would explain a disconnection between the suppression of the growth defect and the restoration of pre-tRNA level would be that decreased pre-tRNA level induces the growth defect while the suppressive actors are acting in the pathway that detects this decrease in pre-tRNA level and translates it into reduced growth. If there is a correlation between suppression of both phenotypes, it will be a good argument for pre-tRNA deficiency to be causing the growth defect.

# *Conclusion & perspectives*

There were two major objectives for this master thesis. The first objective was to uncover new paths of Pol III regulation, using a Pol III mutant (*rpc25-flag*) as a tool. A range of genes were tested for suppression of *rpc25-flag* phenotype but some interesting ones are still to be tested by targeted mutagenesis and complementation of the potentially suppressive gene. In particular, the suppressive effect of genes with disruptive (such as the SAGA subunits *tra1* or *sgf73*) or non-disruptive (*mcs4*, *tms1*) indels/substitutions in the isolated suppressors could be confirmed by this way.

As discussed, additional Northern blot replicates of high quality are required in order to assess the level of pre-tRNA within the suppressors. If there is no restoration of pre-tRNA level within the suppressors, it would be interesting to test the level of other Pol III transcripts within these strains, as they could be the growth limiting Pol III products.

Concerning *slal* overexpression, results show that it causes an increase in pre-tRNA level, but no restoration of growth in the *rpc25-flag* strain and even a worsening of the growth defect when compared with a strain transformed with an empty plasmid. Although, the potential suppressive character of *leu1-32* could be interfering with the interpretation of this result. To demonstrate that *slal* overexpression is not improving *rpc25-flag* growth without interference from *leu1-32*, the same test could be repeated using a different marker to select plasmid conservation, for example uracil autotrophy or antibiotic resistance.

An interesting aspect to investigate would be histones modifications. Chromatin at Pol III transcribed genes share common point with that of Pol II transcribed genes [55]. Different proteins responsible or implicated in different histone modifications were identified as potentially suppressive during suppressors isolation or double mutants creation. It would then be interesting to co-immunoprecipitate Pol III transcribed genes with antibodies against specific histones modification and to compare the enrichment of these ChIP between a wild-type strain and the mutant for the protein potentially implicated in this modification. If significant difference to be found between wild-type and mutant strain for the presence of a particular histone modification at Pol III transcribed genes, the gene deleted in the mutant strain would be required – and probably, directly involved – for this modification at Pol III transcribed gene.

By connecting Ubp8 to SAGA, Sgf73 is involved in H2BK123 deubiquitylation and it is expected that its deletion would increase H2BK123 ubiquitination. If a correlation between *sgf73* deletion and an increase in H2BK123 ubiquitylation at Pol III transcribed genes is observed, other genes could be tested to confirm the link between this modification and SAGA. In particular, the *ubp8* deletion mutant would be interesting to test, as Ubp8 is also required for deubiquitylation [124], but its depletion is not suppressive.

CTD phosphorylation related landmarks on chromatin could also be exploited in order to investigate the recruitment of chromatin modification factors at Pol III transcribed genes by Pol II. There are evidences that at tDNA there is a peak of S5P located upstream of a peak of S2P, similar to what occurs on Pol II transcribed genes (unpublished results, Carlo Yague-Sanz thesis). Other evidences implicate the presence of H3K4 methylation at some tRNA encoding genes by ChIP without H3K36 methylation. However, this study was carried out in human T cell [55] and the situation can very well be different in fission yeast.

The absence of effect on the activity of TORC1 in *rpc25-flag* does not confirm the proposed general implication of pre-tRNA level in TORC1 regulation. As pre-tRNA level in *rpc25-flag* is lower than in the wild-type, phosphorylation of Psk1, which is a good indicator of TORC1 activity, would be expected to be reduced, at least at higher temperature, compared with a wild-type [48]. Our hypothesis to explain this discrepancy, is that the semi-permissive temperature of 34°C does not lead to a sufficient reduction in pre-tRNA level to impact TORC1 in our strain, as it still grows at this temperature. To assess this hypothesis, it could be interesting to perform the same experiment at 37°C, the restrictive temperature for *rpc25-flag*, as it is the condition where its growth defect is the more marked.

To further assess the effect of the flag on Pol III, a complementary experiment would be to perform structural analyses of the complex with protein extracts from the *rpc25-flag* strain. It would allow a precise visualization of the steric clashes and/or structural rearrangements caused by the flag addition on Rpc25.

Finally, although several results still require to be clarified, the main objectives of this project were achieved. Diverse aspects of *rpc25-flag* phenotypes were examined and several actors potentially implicated in Pol III regulation were uncovered. This study may result in a new regulatory pathway for Pol III that link histone modifications by factors usually associated with Pol II to the transcription by Pol III. These pathways would be of particular interest as little is known about Pol III regulation, while its activity impacts a wide range of cellular process. First among the affected processes, the fine-tuning of the proliferative potential of the cell is of critical importance to control cell-fate decision in eukaryotes, from the switch to sexual differentiation in fission yeast [48] to the development of cancers in mammals [127].

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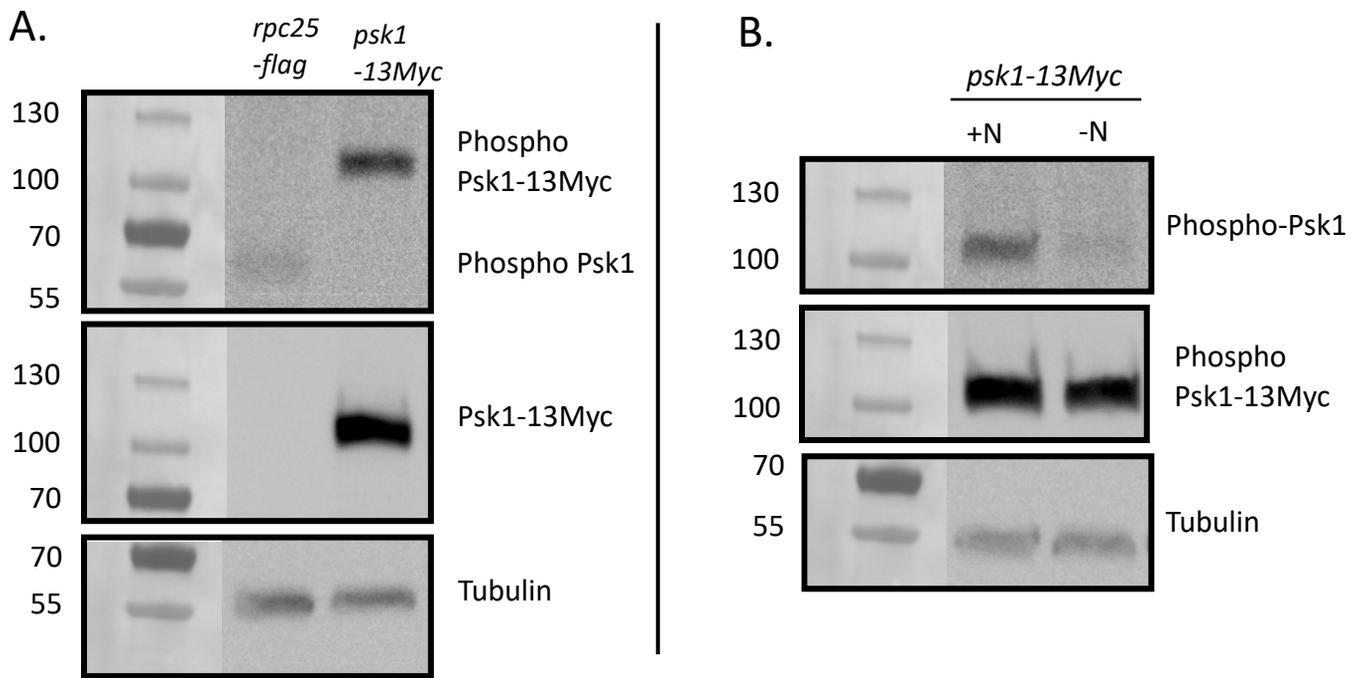
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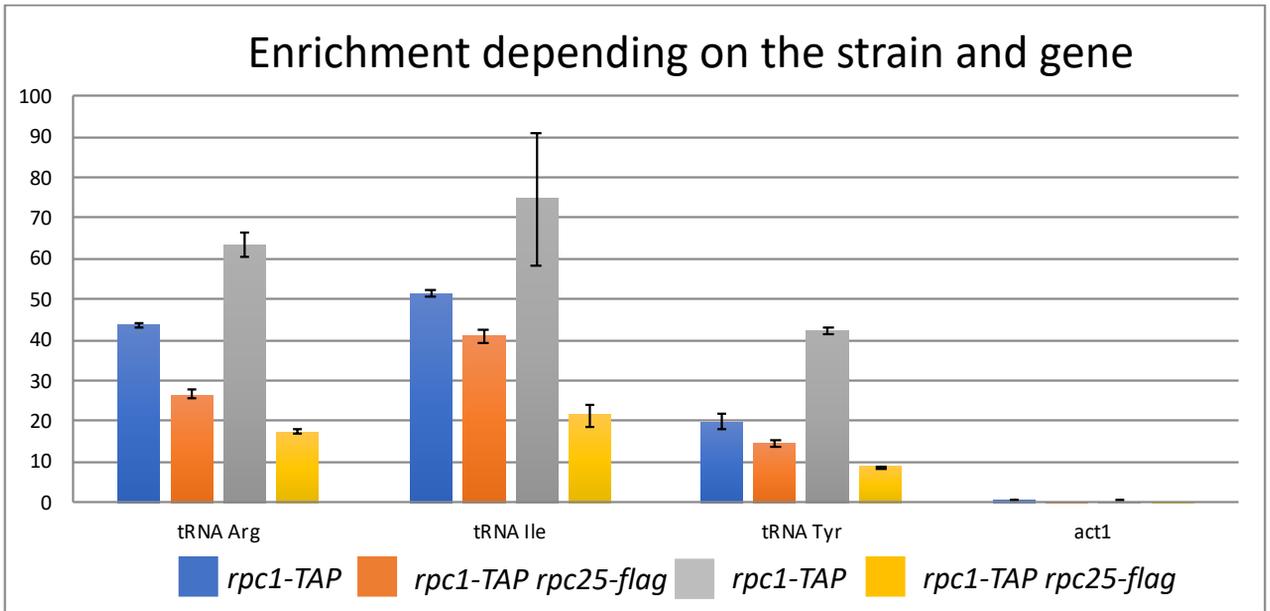
**Supplementary Figure 1: Phospho-Psk1 and Psk1-13Myc.** Cells of *rpc25-flag* and *psk1-13Myc* strains were grown in liquid medium at 32 °C until exponential phase. After TCA extraction, protein extracts were subjected to Western blot analysis using the appropriate antibodies (see Table 2) and revealed by ECL. **A)** Comparison between phospho-Psk1 signal in cultures switched from EMM to EMM without nitrogen for 30 minutes or not. Total amount of Psk1-13Myc and global protein charged **B.** Frameshift induced by the 13Myc addition on Psk1.

## Supplementary Data

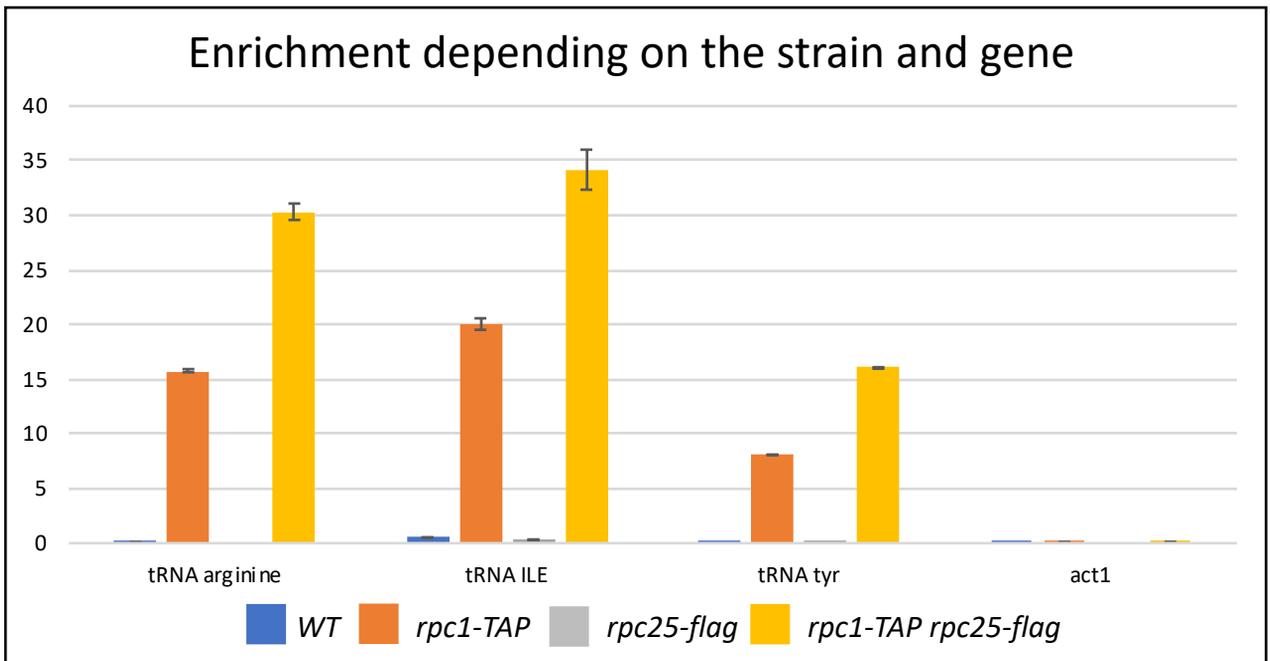
Table S1:PCR probes used in this study

Genotype checked	Position of the probe	Sequence
<b>Mating type</b>	F	AGAAGAGAGAGTAGTTGAAG
	F	ACGGTAGTCATCGGTCTTCC
	R	TACGTTCAGTAGACGTAGTG
<b>rpc25-flag-hphR</b> -	F	GAAGGGTGCACCATCTATTTGC
	R	CGGATGTGATGTGAGAACTGTATCCTAGC
<b>natR</b>		
<b>rpc1-TAP</b>	F	CAACACACTTCAGCAGATTCAC
	R	CGGATGTGATGTGAGAACTGTATCCTAGC
<b>rpc25</b>	F	GGATTGTGATCCACTGTTGT
	R	GCAAATAGATGGTGCACCCTTC
<b>set2::kanR</b>	F	TCGTTTACACTCCGGTACTG
	R	CGGATGTGATGTGAGAACTGTATCCTAGC
<b>atf1::kanR</b>	F	ATGTCCCCGTCTCCCGTCAA
	R	CGGATGTGATGTGAGAACTGTATCCTAGC
<b>sty1::kanR</b>	F	CTTAATCACAGCTACATACCTCC
	R	CGGATGTGATGTGAGAACTGTATCCTAGC
<b>gcn5::kanR</b>	F	CTTGAACATTTCAGCGGTATCG
	R	CGGATGTGATGTGAGAACTGTATCCTAGC
<b>sgf11::natR</b>	F	AGGTCAAATTC AAGTGTGCAAC
	R	CGGATGTGATGTGAGAACTGTATCCTAGC
<b>pmk1::kanR</b>	F	ATGGCCTTCTGTACCAACC
	R	CGGATGTGATGTGAGAACTGTATCCTAGC
<b>set1::natR</b>	F	ACAAAGTAACGTGCGCGG
	R	CGGATGTGATGTGAGAACTGTATCCTAGC
<b>psk1-13Myc</b>	F	TCACCGCCAACAATCATAAG
	R	CGGATGTGATGTGAGAACTGTATCCTAGC
<b>ubp8::ura4</b>	F	TCTATGGGAATTTACCAAGTTTATG
	R	AGAGAAGCTGGTTGGAAGGC
<b>pcr1::kanR</b>	F	TGTATCCACGTCTCTCACAATTC
	R	CGGATGTGATGTGAGAACTGTATCCTAGC
<b>ada2::natR</b>	F	TCAAGTCGTGATTGCAGAGAAG
	R	CGGATGTGATGTGAGAACTGTATCCTAGC

A.



B.



**Supplementary Figure 2 : Co-immunoprecipitation of Rpc1-TAP is specific** *rpc1-TAP* and *rpc1-TAP rpc25-flag* strains were grown in liquid YES medium at 32 °C until exponential phase. After crosslinking and chromatin extraction and sonication, DNA bound to Rpc1-TAP was co-immunoprecipitate. After uncrosslinking, measurement of the relative amount of four genes was performed by Real-time PCR (see corresponding probes **Table S2**). **A)** The average enrichments (percentage IP/total) for five biological replicates, two technical replicates each with error bar corresponding to confidence interval are shown **B)** The average enrichments (percentage IP/total) for two technical replicates with error bar corresponding to confidence interval are shown.

Table S2: Antibodies used in this study

Antigen	Reference
Tubulin	Sigma T5168
Flag	Sigma F3165
TAP	Sigma P1291
Myc	Covance MMS-150P
Phospho-Psk1	Cell Signaling #9206

Table S3: Probes used for Northern Blot

Target	Sequence (5'->3')
LeuCAA tRNA	GTATTACTTGAGTGCTGCGCCATAGACCGC
ARG0.5 tRNA	TAGGAATGAGATGCGCTACCATTGCGCCA
5.8S rRNA	GCAATGTGCGTTCAAAGATT
sla1 mRNA	TTTCGGACACCAACTTACC
	TTCAGAAGCGTTAGATGACC

Table S4: Real time quantitative PCR probes

Target	Position of the probe	Sequence (5'->3')
ARG0.5 tRNA	F	AACCAGGCAAAGGTTGTTAC
	R	TAATTCACCTCCCCAACAACG
Ile0.4 tRNA	F	TGGCAAGAGTGGTGTCCATC
	R	AACCGACTACATCATGCGAC
Tyr0.4 tRNA	F	CAAGCACCGGCTATACAACAC
	R	TGGAAGAGAGCTTGCCTTAGTG
act1	F	CCACTATGTATCCCGGTATTGC
	R	CAATCTTGACCTTCATGGAGCT
5S rRNA	F	TAGGATCGCTGAGAATCCATC
	R	TGGATTA AACACATTGCTTGC
snU6	F	TCTTCGGATCACTTTGGTCA
	R	CCATGCTCTAAACAACGAGT