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## Post-transcriptional regulation of gene expression by the conserved RNAse III Pac1 in S. pombe

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# POST-TRANSCRIPTIONAL REGULATION OF GENE EXPRESSION BY THE CONSERVED RNAse III Pac1 in *S. pombe*.

**Mémoire présenté pour l'obtention**
**du grade académique de master en sciences biomédicales**

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# Post-transcriptional regulation of gene expression by the conserved RNAse III Pac1 in *S. pombe*.

ENGELS Manon

Abstract

**Background.** RNAses are involved in the degradation of RNA, one of the mechanisms used by cells for the post-transcriptional regulation of gene expression. Pac1 is a type III RNAses, which target specific stem-loops within RNA molecules for endonucleolytic cleavage. How Pac1 activity can regulate gene expression is still largely unknown. To address this question, a forward genetic screen for suppressors of a thermosensitive mutant allele of *pac1* was previously performed. It identified a mutation in an intron of *nda3* (a gene encoding for β-tubulin) as a potential suppressive mutation of Pac1 deficiency. **Aim.** We aim to understand how a mutation in a single intron can suppress a growth defect associated with decreased Pac1 activity. **Methods.** Given that the suppressive mutation in *nda3* is within an intron, we hypothesized that the growth defect and the suppression relate to the splicing of that intron. To test this hypothesis, we generated strains in which that specific intron was removed and assessed whether it could suppress Pac1 deficiency. We also used RT-qPCR and western blot assays to measure the levels of intron retention, mature mRNA, and β-tubulin expression. **Results.** Supporting the link between Pac1 activity and *nda3* splicing, intron retention was observed in the Pac1 thermosensitive mutant. In addition, the complete deletion of *nda3* third intron partially suppressed the growth defect associated with Pac1 deficiency, albeit not to the extent of the original suppressive point mutation in *nda3* intron. While this phenotypic suppression could not be directly correlated with to patterns of β-tubulin protein expression, Pac1-deficient cells were found sensitive to TBZ, a microtubule depolymerizing agent, which is indicative of cytoskeleton defects. **Conclusion.** Together, these observations suggest that (1) Pac1 activity is generally important for efficient splicing, but this is probably an undirect effect that relies on Pac1 known function in snRNA biogenesis; and (2) efficient splicing of *nda3* is essential to β-tubulin function. However, the exact mechanism by which the *nda3* mutation within the intron is able to suppress the growth defect of *pac1-ts* remains unclear because it is not functionally equivalent to fully removing the intron, and also because the retention of the third intron is not suppressed by the original mutation, even if the growth defect is.

**Key words**: Pac1, post-transcriptional regulation, *nda3*, splicing, *S. pombe*.

Mémoire de master en sciences biomédicales

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# 1. Abbreviations

| | |
|---|---|
| °C | Celsius degre |
| 5’ss | 5’ splice site |
| A | Ampere |
| AB | Antibody |
| BS | Branch site |
| cDNA | Complementary DNA |
| CI95% | Confidence interval at 95% |
| CRISPR/Cas9 | Clustered Regularly Interspaced Short Palindromic Repeats / CRISPR associated protein 9 |
| Ct | Threshold cycle |
| Cy5 | Cyanines 5 |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxyribonucleotide triphosphate |
| dsRBD | Double-stranded RNA binding domain |
| dsRNA | Double-stranded RNA |
| IgG | Immunoglobulin G |
| kDa | Kilo Dalton |
| ME | Malt extract |
| Mg | Milligram |
| mL | Milliliter |
| mRNA | Messenger RNA |
| mv | Millivolt |
| ncRNA | Non-coding RNA |
| nm | Nanometer |
| NP-40 | Nonidet P-40 |
| NPC | Nuclear pore complex |
| Nt | Nucleotides |
| OD | Optical density |
| ORF | Open reading frame |
| PBS | Phosphate-buffered saline |
| PCR | Polymerase chain reaction |
| PolyA | Polyadenylation |
| PPT-3’ss | Polypyrimidine tract / 3’ splice site |
| Pre-mRNA | Pre-messenger RNA |
| qPCR | Quantitative PCR |
| RBP | RNA binding protein |
| RNA | Ribonucleic acid |
| RNAP II | RNAP polymerase II |
| RNAse | Ribonuclease |
| Rpm | Revolution per minute |
| rRNA | Ribosomal RNA |
| RT | Reverse transcription |
| SDS | Sodium dodecyl sulfate |
| sgRNA | Single guide RNA |
| siRNA | Small interfering RNA |
| snoRNA | Small nucleolar ARN |
| snRNA | Small nuclear RNA |
| snRNP | Small nuclear ribonucleoprotein |
| TBZ | Thiabendazole |
| UTR | Untranslated transcribed region |
| V | Volt |
| WT | Wild type |
| YES | Yeast extract supplemented |
| µL | Microliter |

# 2. Introduction

The goal of this work is to study how gene expression can be regulated by an RNAse in a post-transcriptional manner. Because the RNA level is tightly regulated by post-transcriptional regulations to have the appropriate number of proteins needed in the cell, mRNA expression is not in perfect correlation with the number of proteins produced (Corbett 2018). The RNA levels are regulated by several mechanisms including the RNA transport, processing, and modifications, but also through the stability of the RNA molecules. RNases are involved in the modulation of the RNA stability by degrading mRNA and preventing them from being translated into proteins (Pradeep Kumar Singh et al. 2021).

To study post-transcriptional regulations, we chose the fission yeast *Schizosaccharomyces pombe*, which is a very useful model organism for the study of post-transcriptional regulation. It was isolated in 1890 and first described in 1893 by Paul Lindner (Lindner 1893). *S. pombe* is a unicellular eukaryote notably used to study the regulation and conservation of the eukaryotic cell cycle (Vyas et al. 2021), the differentiation, the RNA splicing (Sabatinos and Forsburg 2010) and for the production of heterologous proteins (Johnson and Echavarri-Erasun 2011), among other utilizations. There is lots of common mechanisms and elements with humans, including genes structures and control of gene expression. The fission yeast is also easy to handle genetically, grows as haploid or diploid faster than most other eukaryotes model.

The RNases can be classified into different groups and families depending on their activity (endo- or exo-ribonuclease) and mode of action. The RNase III family of ribonuclease is characterized by an endonucleolytic activity specific to structured RNAs. They play a role in the regulation of gene expression, by cleaving RNAs (Catala and Abou Elela 2019). In *S. pombe*, there are two RNase III proteins: Dicer and Pac1. The first one has a well-studied role in the RNA interference by the cleavage of double strand RNAs and production of siRNAs. The putative roles of Pac1 in the post-transcriptional control of gene expression are much more elusive. This study aims to uncover some of them.

## 2.1. The post-transcriptional regulation at the RNA level

The regulation of gene expression at the post-transcriptional level occurs after the transcription of DNA into pre-mRNA and before the translation of mature mRNA into a protein. Many regulatory layers exist for post-transcriptional regulation at the mRNA level in eukaryotes.

Such regulations can also be co-transcriptional. In that case, the regulation takes place whilst the RNA is being transcribed, when it is still tethered to the DNA by the polymerase. The post-transcriptional regulation, on the other hand, takes place after the completion of the RNA synthesis. For instance, the RNA capping (the addition of a 7-methylguanosine cap at the 5' end of the transcript to protect it from the ribonuclease degradation) occurs as soon as the first few mRNA nucleotides are transcribed. On the 3' end, the cleavage and polyadenylation complex adds a polyA tail to the nascent transcript and triggers the termination of the transcription and the release of the polymerase. The capping and polyadenylation both contribute to the maturation of pre-mRNAs (Dower et al. 2004).

Another regulatory mechanism specific to eukaryotes is the RNA splicing, which can occur either co-transcriptionally or post-transcriptionally. It contributes to the gene regulation process, the protein diversity, and the phenotypic complexity in eukaryotes. The introns present

in most proteins-coding genes, but also in ncRNA, are transcribed into the pre-mRNA. To form the mature mRNA, which will later be translated into a functional protein, introns need to be removed and exons (coding sequences) need to ligate together (Plaschka, Newman, and Nagai 2019).

This process implicates the spliceosome, a dynamic multimegadalton enzymatic complex. It is composed of five snRNAs and many proteins. The snRNAs (U1, U2, U4, U5 et U6) associate with proteins to compose the snRNPs complexes. Each snRNAs involved in the splicing has specific roles in the spliceosome to carry out the splicing. Some other non-snRNPs proteins complexes are also needed for a correct splicing of pre-mRNAs (Lerner et al. 1980; Plaschka et al. 2019; Yan, Wan, and Shi 2019).

The splicing factors are proteins that bind to signals of recognition located both in the intron and in the flanking exons. Three mains splicing signals are known to be present in eukaryotes: the 5' splice site (5'ss) located at the 5' end of the intron (Figure 1b), the polypyrimidine tract / 3' splice site (PPT-3'ss) located at the 3' end of the intron (Figure 1d) and the branch site (BS) located upstream of the PPT-3'ss (Figure 1c). Splicing factors bind to these splicing signals to initiated the spliceosome assembly and begin the splicing of the pre-mRNA (Plaschka et al. 2019; Schwartz et al. 2008).The introns are removed and the exons are ligated together by two consecutives phosphoryl-transfer reactions (Plaschka et al. 2019) (Figure 1a).

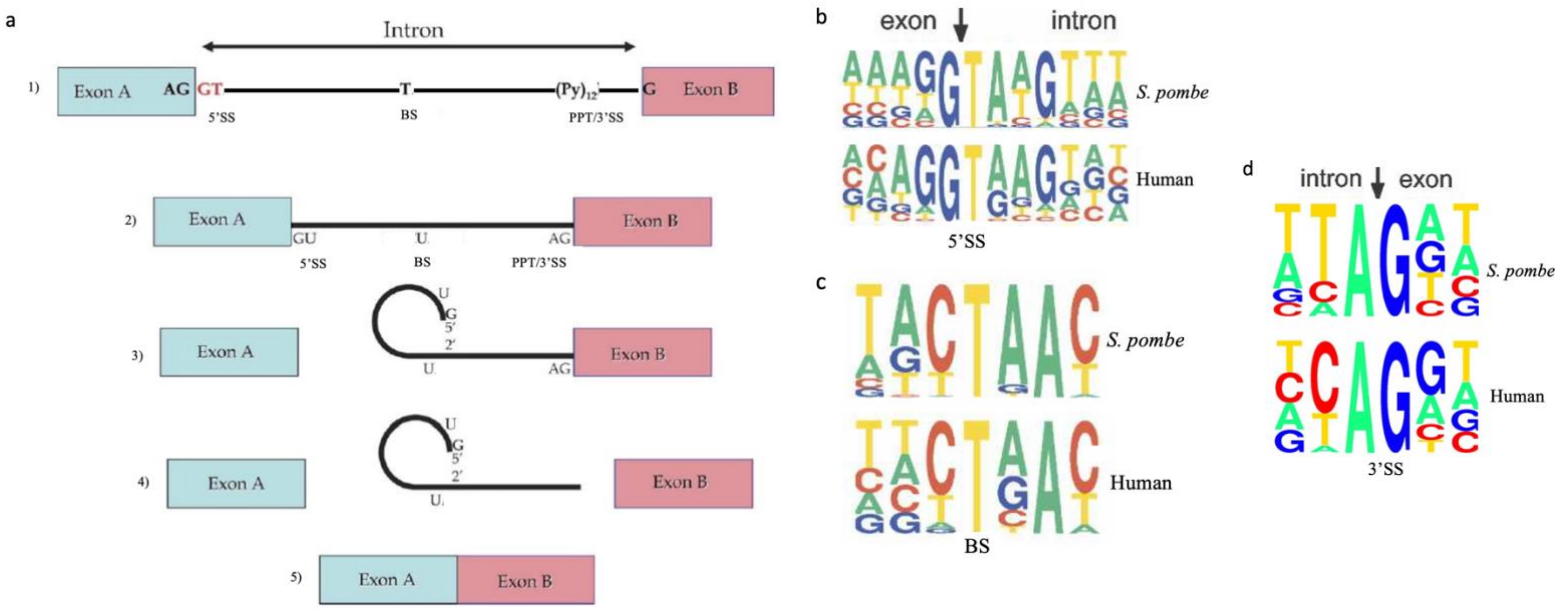



**Figure 1: Splicing mechanisms and features. (a)**. Splicing signals positions on the introns (adapted from *(Wessagowit et al. 2005)*. **(b)**. Consensus 5'ss signals of *S. pombe* and human (adapted from Schwartz et al. 2008). **(c)**. Consensus BS signals of *S. pombe* and human (adapted from Schwartz et al. 2008). **(d)** Consensus 3'ss signals of *S. pombe* and human (adapted from Schwartz et al. 2008).

Both the transcription and the splicing of introns are costly processes that use resources from the cells. To balance that cost, their presence in the genome confers many advantages. One of them is the increased proteome diversity as a result of alternative splicing (Juneau et al. 2006). In budding yeast, introns promote cell survival in starvation conditions (Parenteau et al. 2019) and they regulate the RNA and protein abundance of their genes of origin (Juneau et al. 2006).

The spliceosome interacts closely with proteins involved in mRNA metabolism (transcription, capping, polyadenylation, RNA export and nonsense mediated decay) (Juneau et al. 2006).

The modulation of the RNA localization is used by cells to regulate their proteins expression level. The control of the localization of mRNA allows the corresponding protein to be produced in the subcellular compartment of interest, thus reducing the cost associated with the transport of proteins. mRNA can also be localized in compartments or organelles that prevent them of being translated or that allow them of being degraded. Their trafficking is controlled by "zipcode" (*cis*-localization element) together with RNA binding proteins (RBPs). Also, non-canonical modes of mRNA transport seem to exist (Das et al. 2021). Finally, the correct export of mRNA depends on the different steps of transcription (gene activation, splicing, 3' end cleavage, NPC recruitment) but also on subsequent steps such as mRNP modification or translation. The export only occurs if these steps happened correctly. If the mRNA produced after processing is aberrant, it is blocked in the nucleus and will be degraded (García-Oliver, García-Molinero, and Rodríguez-Navarro 2012).

The RNA level regulation partially goes through its stability in the cell, including the degradation, one of the mechanisms for post-transcriptional regulation. This process is taken care of by the RNases. The RNAses could be endoribonucleases or exoribonucleases depending on their mode of action.

They act in accordance with the cell environment and carry important roles in the cell: the maturation of ncRNAs, the recycling of ribonucleotides, the RNA surveillance (destruction of aberrant RNAs). The degradation process depends on the structure of RNAs for the stabilization of RNAses, on the presence or not of ribosomes that may hide some RNA segments sensitive to RNAses, on polyA stretches that are substrates to some RNAses and on some *trans*-acting factors that hide or expose RNA sites (Arraiano et al. 2010).

RNA editing is also one of the mechanisms for post-transcriptional regulation of gene expression. This mechanism is used by the cell to modify the RNA sequence after the transcription, by the insertion or deletion of a base or by the substitution of a base by another one, mostly adenosine to inosine and cytidine to uracil. It affects the activity, stability, localization, function, splicing and translation of mRNAs. It happens either co-transcriptionally or post-transcriptionally. It is regulated by cellular stimuli or environmental conditions (Gott and Emeson 2000; Licht and Jantsch 2016).

## 2.2. The Pac1 RNase III ribonuclease

*pac1* is an essential double-stranded RNA endonuclease encoding gene present on the chromosome II of *S. pombe* and conserved up to humans (Drosha). The gene encodes for a polypeptide that has two different domains. The first one is the double-stranded RNA-binding domain (dsRBD) of approximately 70 residues. The second one is the catalytic domain of 150 residues that carries the ribonuclease III activity (UniProt, 2022).

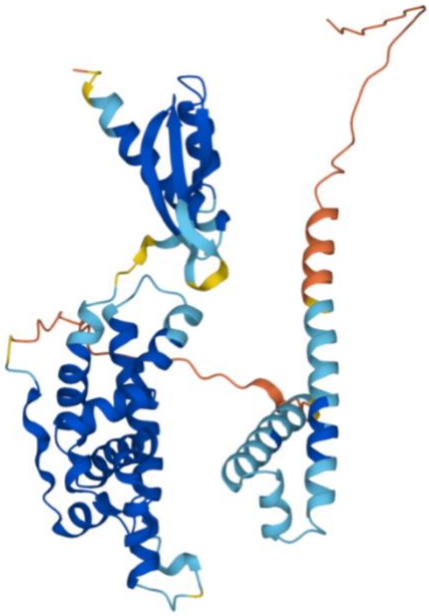


**Figure 2**: Predicted 3D conformation of Pac1 protein (AlphaFold). Colors indicate the degree of confidence in the model: from very high (blue) to very low (orange).

*pac1 (pat1 compensation 1)* was originally discovered as a multicopy suppressor of the *pat1* temperature-sensitive mutation that induces uncontrolled meiosis in fission yeast (Iino, Sugimoto, and Yamamoto 1991). This indicates that the overexpression of *pac1* compensates for the loss of function of Pat1, an essential kinase repressing meiosis when cells grow as haploids (Cipak et al. 2012).

As an RNase III, Pac1 is involved in the maturation of structured RNAs. It cleaves dsRNAs in two staggered cuts by specifically targeting stem-loop regions in RNAs. During the co-transcriptional folding of mRNAs, Pac1 cleavage on the nascent stem-loop can create entry points for 5'-3' and 3'-5' exonucleases and causes the degradation of the mRNA being transcribed (Figure 3) (Yague-Sanz et al. 2021). Some indirect roles of Pac1, via the effect on snRNA, in the splicing of pre-mRNA has also been demonstrated (Potashkin and Frendewey 1989) and Pac1 triggers transcription termination of snRNA and specific protein-coding genes (Yague-Sanz et al. 2021). Finally, considering the link with Pat1, Pac1 is presumably involved in controlling meiosis and sexual differentiation. However, the mechanisms coupling Pac1, and sexual differentiation and meiosis, has remained unknown. This is currently studied in the GEMO lab, although this question is not the subject of this master thesis.

More specifically, Pac1 is involved in 3 different types of maturation of RNA.

The 3' end of the 35S rRNA exhibit a highly conserved stem-loop region, which is targeted by Pac1 that allows the maturation of the 35S rRNA precursor into the mature 25S and 18S rRNAs (Ivakine et al. 2003). However, the interaction between Pac1 and the stem-loop structure does not seem to lead to the termination of the transcription (Yague-Sanz et al. 2021).

Pac1 is also involved in the maturation and transcription termination of some snoRNAs. In particular, the snU3, snU32 and snR88 are affected by Pac1. The cleavage targets the stem-loop structures at the 3' end of the snoRNAs precursors (Nabavi and Nazar 2010b; Yague-Sanz et

al. 2021). Pac1 also has roles in the maturation of the 5'end of C/D box snoRNAs (Yague-Sanz et al. 2021).

Pac1 triggers transcription termination of snRNAs. Indeed, snU1, snU2, snU4, snU5 showed a strong association with Pac1, which binds to the stem-loop region at the 5' end of these transcripts (Nabavi and Nazar 2010a; Yague-Sanz et al. 2021).

Besides these roles, the full breadth of Pac1-dependent regulation remains largely an uncharted territory.

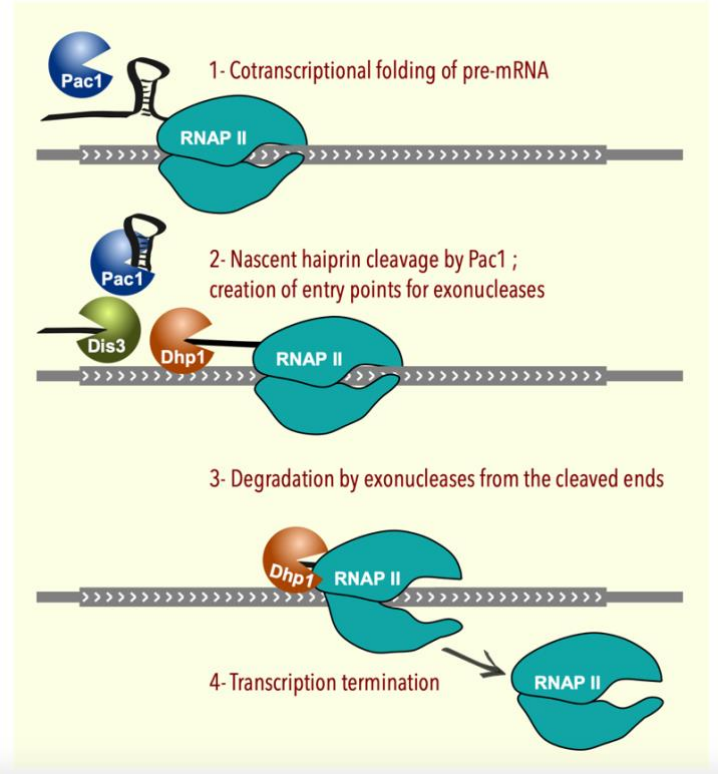



**Figure 3**: Model of Pac1-mediated gene repression. The cleavage of the nascent mRNA mediated by Pac1 create entry points for exonucleases to degrade the pre-mRNA and this process cause transcription termination *(Yague-Sanz et al. 2021).*

## 2.3. Identification of *pac1* thermosensitivity suppressors.

As a strategy to discover news roles of *pac1*, a suppressor screening was previously performed by C. Yague-Sanz and M. Robichaud (unpublished work, F. Bachand's laboratory, Canada). They took advantage of a previously characterized thermosensitive allele of *pac1* (*pac1-ts*) containing a mutation (G1024A) affecting the Pac1 dsRNA-binding domain (Potashkin and Frendewey 1990). The *pac1-ts* mutant exhibits a reduction in the expression level and longer transcripts of several snRNAs, causes defects in snRNAs processing and disables cellular grows at high temperature. They used the *pac1-ts* strain and spread it on agar plates. After letting it grow at restrictive temperature, a few colonies grew despite the original mutation, suggesting that a random additional mutation appeared, which suppresses the thermosensitivity. This has also been done by placing the strain in 96-wells plates and letting it grow in liquid at 37°C for a few days. Visible growth could be observed in only one of the *pac1-ts*-containing wells. The

colonies that were able to grow at restrictive temperature are suppressors of the *pac1-ts* thermosensitive phenotype.

After isolating the suppressors, their whole genome was sequenced to identify which mutations were present. Three different mutations were detected, each of them in a different suppressor strain (S1, S2 and S3) (Table 1). Note that one single mutation was observed by suppressor, which is expected since the screen did not rely on any mutagenetic treatment.

| NAME | GENOTYPE | GENE FUNCTION |
|---|---|---|
| **S1** | *pac1* G1024A / *ams2* G1304A | *ams2*: DNA binding transcription factor |
| **S2** | *pac1* G1024A / *nda3* T216+T | *nda3*: β-tubulin |
| **S3** | *pac1* G1024A / *pdt1* A1094T | *pdt1*: plasma membrane transporter |

**Table 1:** The three identified suppressors of the *pac1-ts* strain and their mutations identified after whole genome sequencing (C. Yague-Sanz and M. Robichaud, unpublished work).

By studying the suppressors of *pac1-ts* and their potential relationship with Pac1 activity and functions, the ultimate goal of this project is to discover new roles of *pac1*. This will allow us to learn more about the post-transcriptional regulation of gene expression by ribonucleases.

## 2.4. The transcription factor Ams2

With the result of the whole genome sequencing (Table 1), the nucleotidic G1304A mutation in *ams2* gene was identified in one strain that was able to suppress the thermosensitive phenotype of *pac1-ts*.

Ams2 is a DNA binding transcription factor that activates the transcription of histone genes. It contain a GATA (zinc finger) motif (Takayama, Shirai, and Masuda 2016) and associates with chromatin (Chen et al. 2003). Histones are proteins that permits the compaction of DNA into the nucleus and have major roles in the regulation of genome functions.

The production of histone is a cyclic phenomenon, with a peak during S phase where the demand is highest. The transcription of the *ams2* gene also peaks during M phase (Takayama et al. 2016) and is responsible for the increased histone transcription in S phase by binding to the promoter region of the core histone genes, the G1 phase being very short in fission yeast. Thanks to Ams2, the synthesis of DNA and the production of histones are synchronized (Takayama and Takahashi 2007). The deletion of *ams2* results in the loss of cycling histone production, in a decrease centromeric histone occupancy, and in chromosome missegregation (Chen et al. 2003).

The G1304A mutation in *ams2*, presumed to be able to suppress the thermosensitive phenotype of *pac1-ts*, results into a cysteine to phenylalanine mutation in the Ams2 zinc finger domain (GATA type) (Figure 4). This domain is necessary for the correct function of *ams2*: it allows Ams2 to bind to the promoter of the histone genes and to activate their transcription (Takayama et al. 2016).

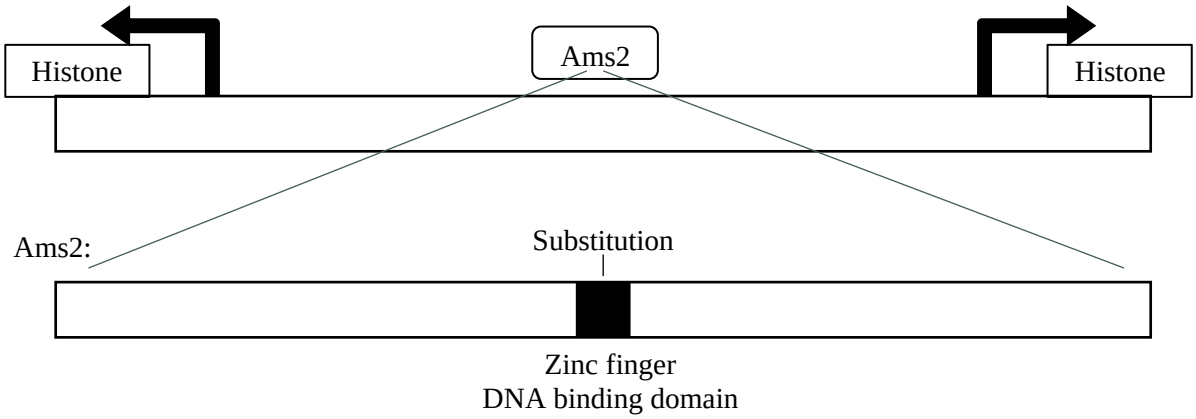



**Figure 4:** Schematic representation of the transcription factor Ams2 localization on the genome and the localization of the mutation in the protein.

## 2.5. The tubulin-encoding gene *nda3*

After the whole genome sequencing (Table 1), a nucleotidic T216+T mutation in the *nda3* gene was identified in a strain (S2) that was able to suppress the thermosensitive phenotype of *pac1-ts*.

The *nda3* gene is localised on the chromosome II of *S. pombe*. It codes for the β-tubulin, an essential protein that associates with α-tubulin to form microtubules, that are the central constituent of the cytoskeleton. The cytoskeleton is involved in the maintenance of cell shape and movement, and in the localization of organelles in the cell. It is also critical for chromosomes segregation.

β-tubulin and α-tubulin associate in heterodimers to form the microtubules (Figure 5). Therefore, stochiometric amount of β-tubulin and α-tubulin is critical for efficient microtubule formation. The ratio between the α- and the β-tubulin is known to be important for normal growth. For instance, an excess of β-tubulin can cause microtubule disassembly and cell death (Alvarez et al. 1998).

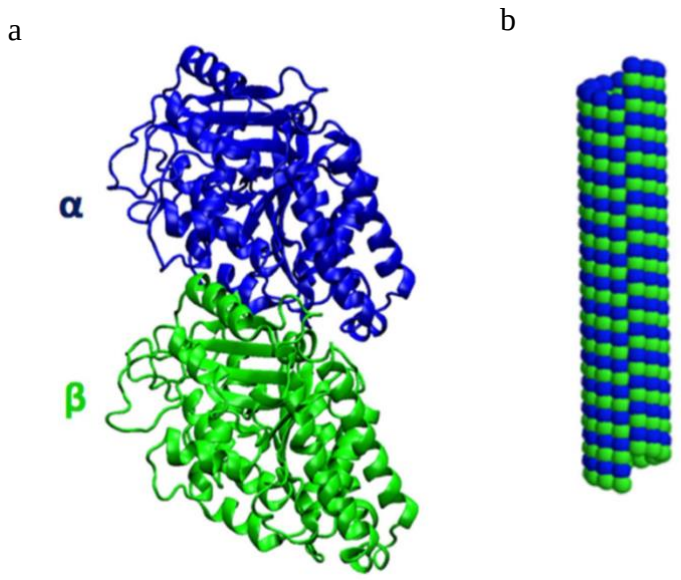



**Figure 5**: Tubulin dimer conformation **(a)** and formation of microtubules **(b)** (adapted from *(Bassen et al. 2016)*.

*nda3* has fives introns, four of them are located within the 5'end of the gene. Intriguingly, the *nda3* T216+T mutation localizes within the third intron (Figure 6). This raises questions about the mechanisms by which the mutation suppresses *pac1-ts* thermosensitivity.

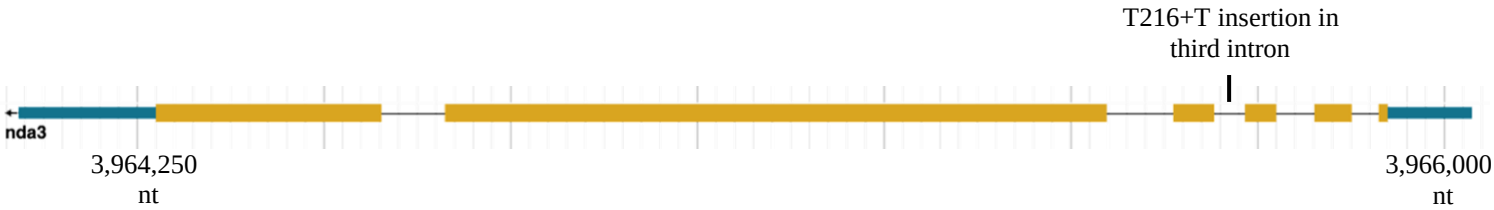



**Figure 6**: Schematic representation of the localization of the mutation in *nda3* gene (1,750 nucleotides in size). Exons are represented in orange and the 3' and 5' UTR in blue. (PomBase, 2022).

## 3. Objectives

The general objective of this study is to fully characterize suppressors of *pac1-ts*. This will possibly lead us to discover new roles of *pac1* and, ultimately, to better understand the post-transcriptional regulation of gene expression by proteins of the RNase III family.

Specifically, we aim to functionally characterize the potential *nda3* suppression of *pac1-ts* thermosensitivity, with the purpose of understanding the links between *pac1* and *nda3*. This will lead us to evaluate the possible mechanism linking the ribonuclease Pac1 with β-tubulin expression. Also, we aim to understand how the addition of a single nucleotide within an intron of *nda3* is able to suppress the *pac1-ts* thermosensitive phenotype.

# 4. Materials and Methods

## 4.1. Growth conditions and strains

*Schizosaccharomyces pombe* (Table 2) were cultured in YES (Yeast Extract Supplemented) medium (YES broth, Formedium, PCM0310) at 25°C except when otherwise stated.

| NAME | ID | SOURCE | GENOTYPE |
|---|---|---|---|
| 972 h- | yDH94<br>yDH51<br>972 | Lab stock | h- |
| *nda3 UCU-ACC* | yDH2021 | (Finet et al. 2022) | nda3 UCU-ACC, h- |
| wild type | 2100<br>FBY13<br>PSY2928 | (Bachand and Silver 2004) | h+, ade6-210, ura4-D18, leu1-32, his3-D1 |
| *pac1-ts* | 2107<br>FBY2604 | (Yague-Sanz et al. 2021) | pac1 G1024A, h+, ade6-210, ura4-D18, leu1-32, his3-D1 |
| *pac1 G1024A* | 2120 | This study | pac1 G1024A, h90 |
| *pac1-ts nda3 T216+T* | 2132<br>FB2746 | This study | pac1 G1024A, nda3 T216+T, h+, ade6-210, ura4-D18, leu1-32, his3-D1 |
| *nda3 [Δintron3]* | 2173 | This study | nda3 [Δintron3], h+, ade6-210, ura4-D18, leu1-32, his3-D1 |
| *pac1-ts nda3 [Δintron3]* | 2176 | This study | pac1 G1024A, nda3 [Δintron3], h+, ade6-210 |
| *nda3 T216+T* | 2181 | This study | nda3 T216+T, h+, ade6-210 |

**Table 2**: *S. pombe* strains used in this study.

## 4.2. Growth curves

Cells at $OD_{595nm}$ = 0,5 were collected and diluted at $OD_{595nm}$ = 0,01. 150 µL of cells were incubated with 6 wells/conditions in a 96-wells plate at 37°C for +/- 64h in a Bioscreen device (BioTek EPOCH 2 microplate reader). The $OD_{595nm}$ was measured every 15 minutes and averaged across replicates. The standard deviation and the 95% confidence interval were also calculated.

## 4.3. Spot assays

Growing cells at $OD_{595nm}$ = 1 were 5-fold serially diluted. 5 µL of each concentration was spotted on YES plates. Plates were incubated at 26°C, 32°C or 37°C for 3 days before pictures were taken.

## 4.4. Western blot

25 mL of cells were cultured at 26°C or 32°C. When $OD_{595nm}$ = 0,5, the protein extraction was done using a mechanic extraction with zirconium beads. Briefly, harvested cells were pelleted and 1 mL of NP-40 buffer (with IGEPAL CA630, Sigma n°56741, a non-ionic non-denaturing detergent), was added. Afterwards, 1 mL of zirconium beads is added, and the mix is homogenised using the FastPrep machine (homogeniser for lysis of biological samples) set at 5,5 m/s during four cycles of 30 seconds-2 minutes rests in the cold room. The sample is washed using NP-40 buffer and the supernatant is collected. The samples are next prepared using the Cy5 Total Protein Normalization technique (Amersham Quick Stain Kit RPN400). Then, they are boiled for 5 minutes at 95°C after addition of the loading buffer mixed with β-mercaptoethanol (BioRad Laemmli Sample Buffer #1610737; 2-Mercaptoethanol #1610710).

15 µL of protein were loaded on a Bio-Rad mini-PROTEAN TGX gel 4-15% (reference n°4561083) and run for 10 min at 100 mv and then for 25 minutes at 140 mv. Proteins were then transferred to a nitrocellulose membrane using the Bio-Rad Trans-Blot Turbo Transfer system on Mix Molecular weight at 1.3A/25V for 7 minutes. The membrane was blocked with Skin Milk Powder (5%) (Millipore) during 1 hour at room temperature. After that, the membrane was incubated with the primary antibody (anti-β-tubulin 1/1000 [Agrisera AS21 4556] or anti-α-tubulin 1/2000 [Sigma T5158]) for 1 hour at room temperature. Then, the membrane was washed 3 times with PBS-Tween (Dulbecco's Phosphate Buffered Saline - Tween at 0,05%). The membrane was incubated with the secondary antibody (anti-mouse IgG perox 1/2000 [GE NA031V] or anti-rabbit IgG perox 1/2000 [GE NA934V]) for 1 hour at room temperature. The membrane was washed twice with PBS-Tween (0,05%) and once with PBS.

The revelation was done using the PerkinElmer Western Lightning Plus-ECL on an Amersham ImageQuant 800 machine. The ImageQuant TL software was used for the quantification of protein levels.

## 4.5. RT-qPCR

Total RNA extraction is done using the classical hot phenol protocol as previously described (Schmitt, Brown, and Trumpower 1990). Three successive phenol extraction are followed by one ethanol precipitation and two ethanol washes. The total RNA obtain is purified on column.

Then, complementary DNA (cDNA) was synthetized by a reverse transcription (RT) reaction. The RNA is mixed with a mix of dNTP, random primer, a buffer, the RT enzyme, and water. The mix is put 10 min at 25°C, 2 hours at 37°C and 5 minutes at 85°C.

The cDNA obtain was used to do quantitative PCR reaction. The relative quantity of RNA present in the samples was calculated using the ΔΔCt method on the SyberGreen (Roche) fluorescent signal. The different oligonucleotides used for the RT-qPCR are listed in Table 3.

| NAME | ID | SEQUENCE |
|---|---|---|
| *act1* + 948 F | 739 | CCACTATGTATCCCGGTATTGC |
| *act1* + 948 R | 740 | CAATCTTGACCTTCATGGAGCT |

| | | |
|---|---|---|
| *mfs2* ORF F | 3622 | TGCAATGATTCAGGCAGCTACTC |
| *mfs2* ORF R | 3623 | GTAGTTAATGATGTACACCACAGTC |
| *nda3* exon 3 F | 3899 | GAGCATGGTTTGGATTCAGC |
| *nda3* intron 3 F | 3900 | ATGTAGGTCTATTTTGCTTG |
| *nda3* intron 3 R | 3901 | CTCGTTAAAATAAACGTTCA |

**Table 3**: Oligonucleotides used for the RT-qPCR.

## 4.6. CRISPR/Cas9

The CRISPR/Cas9 experiments were done as previously described (Zhang et al. 2018), using the split-ura4 system. The PCR template for generating the sgRNA insert was generated from pDH823 by NotI digestion and sgRNA primer n°3898 and n°3102 were used. The gapped plasmid was generated from pDH8822 by NotI digestion, and the donor DNA used are n°3896 and n°3897. The different oligonucleotides used for CRISPR/Cas9 are listed in Table 4. The clones obtained were analysed by PCR using oligonucleotides presented in Table 5.

| NAME | ID | SEQUENCE |
|---|---|---|
| Split-ura4 R | 3102 | CATCTGGTGTGTACAAAATTG |
| Donor DNA *nda3* Δintron F | 3896 | AAGGTCTACCATTGCTGATGAGCATGGTTTG GATTCAGCTGGAATATATCATGGAACTTCTG AGGCACAACACGAGCGATTGAACGTTTA |
| Donor DNA *nda3* Δintron R | 3897 | TAAACGTTCAATCGCTCGTGTTGTGCCTCAG AAGTTCCATGATATATTCCAGCTGAATCCAA ACCATGCTCATCAGCAATGGTAGACCTT |
| sgRNA *nda3* | 3898 | ATAGTTGCTGTTGCCAAAAAACATAACCTGT ACCGAAGAA-TTTGGATTCAGCTGGAATGT-gttttagagctagaaatagcaag |

**Table 4**: Oligonucleotides used for the CRISPR/Cas9

This technique was used in the WT strain (n°2100) to obtain the *nda3 [Δintron3]* (n°2173) strain.

## 4.7. Mating

Mating is the crossing of two strains of opposite mating type (h+ and h-). To obtain the strain pac1-ts nda3 *[Δintron3]* (n°2176), the *nda3 [Δintron3]* (n°2173) and *pac1 G1024A* (n°2120) strains were crossed. To obtain the strain *nda3 T216+T* (n°2181), the *pac1-ts nda3 T216+T* (n°2132) and 972 h- (n°94) strains were crossed. Both pairs of strains were spread together on ME (malt extract) medium to induce sexual differentiation for 2-3 days. Then, the presence of

tetrad was checked under the microscope, and tetrads are digested overnight in a thermomixer at 29°C and 800 rpm using β-glucuronidase-arylsulfatase enzyme (Roche 10127060001). Spores released from the digested tetrads are then washed using SDS 1% and spread on agar plates with YES medium for 3-4 days before being replicated on YES supplemented with kanamycin. Then, the colonies are checked for the presence of the expected mutation by PCR using oligonucleotides listed in Table 5.

| NAME | ID | SEQUENCE |
|---|---|---|
| *nda3* exon 1 F | 3755 | TTCCTCCTAACGAAACTCACC |
| *nda3* exon 4 R | 3756 | AGGTTCCACAACGGTATCAG |
| *nda3* intron 3 R | 3901 | CTCGTTAAAATAAACGTTCA |

**Table 5**: Oligonucleotides used for the PCR reaction.

# 5. Results and Discussion

## 5.1. Validation of presumed suppressive mutations

To validate the identity of the suppressing mutation in each *pac1-ts* suppressors, a Sanger sequencing experiment was performed on the following strains: a wild-type (WT), the thermosensitive (*pac1-ts*), and the two suppressors that were able to grow after repatching: S1 and S2. The S3 strain was not able to grow after repatching it. As expected, the *pac1-ts* mutation (G1024A) is present in the *pac1-ts*, the S1 and the S2 strains (Figure 7a). The *ams2* mutation (G1304A) is not present in any strains (Figure 7b). Finally, the *nda3* mutation (T216+T) is only present in the S2 strain (Figure 7c), as expected.


**Figure 7: Sequencing of *pac1*, *ams2*, *nda3* and *pdt1* in WT, *pac1-ts*, S1 and S2 strains. Wild-type nucleotide is coloured in green, and the mutated one is in red. Numbering indicates the nucleotide position relative to**

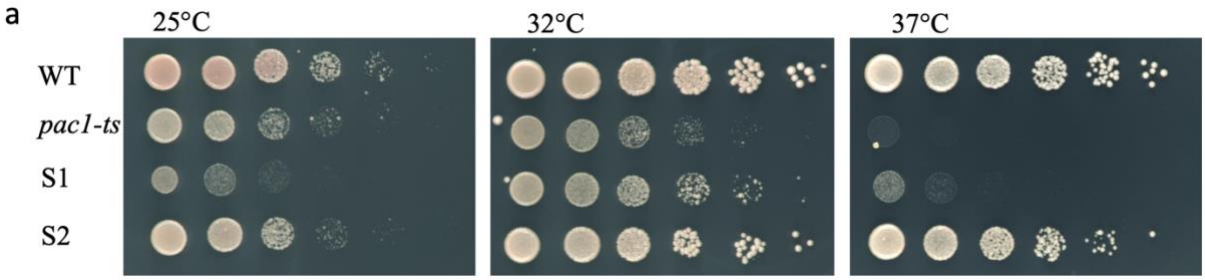
**the ORF of the gene. (a).** Sequencing of *pac1*. **(b).** Sequencing of *ams2*. **(c)**. Sequencing of *nda3*. **(d)**. Sequencing of *pdt1*.

Based on these results, we have confirmed that the S2 strain contains the original *pac1-ts* mutant and confirmed the additional mutation in *nda3*, as originally determined by whole genome sequencing. We will therefore continue to work on it. For the S1 strain, that we thought had a mutation in the *ams2* gene (Table 1), we could not confirm the original suppressive mutation. We checked for the *pdt1* mutation (A1094T) previously identified in S3 by whole genome sequencing, as we hypothesized that the S1 and S3 strains could have been switched at some stage, but the result was also negative (Figure 7d). Without further characterization, we decided not to continue to work with that S1 strain.

## 5.2. Validation of the suppression of the *pac1-ts* thermosensitivity

To evaluate the suppressive potential of the S1 and S2 mutants, their growth was analysed by spot assays and in liquid at permissive and restrictive temperatures. In the spot assays (Figure 8a), the WT strain grows well independently of the temperature. The *pac1-ts* strain grows normally at 26° and has a slight growth defect at 32°C but its growth is stopped at 37°C. This is expected considering the *pac1-ts* is a well-characterized thermosensitive strain (Potashkin and Frendewey 1990; Yague-Sanz et al. 2021). In the S1 strain that has the *pac1-ts* mutation, we observed a slightly increased growth at 32°C and at 37°C compared to the *pac1-ts* strain. The S1 strain also seems to be cold-sensitive as the growth is delayed at 26°C compared to both the WT and the *pac1-ts* strain. Finally, a clear suppression of the thermosensitivity in the S2 strain that contains the *pac1-ts* and the *nda3 T216+T* mutations is visible at 32°C and, most strikingly, at 37°C.

Consistent with the results of the spot assays, growth curves measured in liquid medium confirm that the S2 strain is able to grow at restrictive temperature despite the presence of the *pac1-ts* mutation, while the S1 strain barely suppresses the thermosensitivity (Figure 8b).



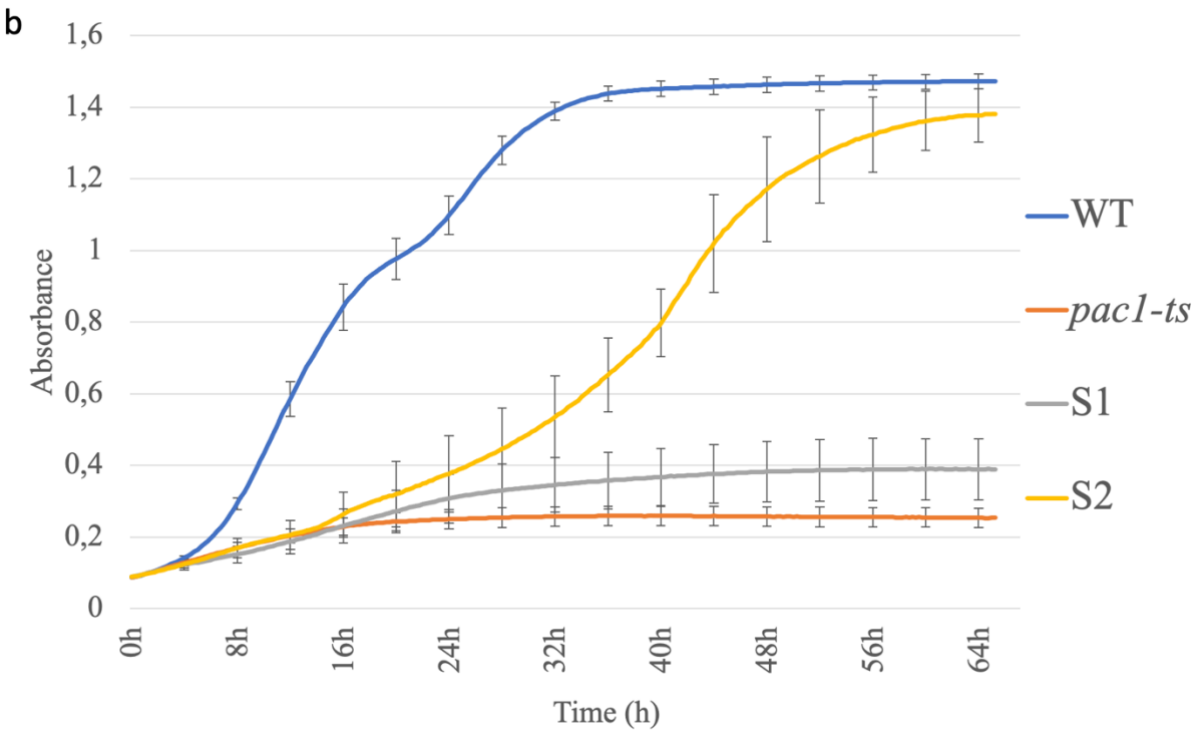



**Figure 8: Thermosensitivity of *pac1-ts* and evaluation of the phenotype of S1 and S2. (a)**. Spot assays of WT, *pac1-ts*, S1 and S2 at 25°C, 32°C and 37°C after 3 days on YES plates. Cells were 5-fold diluted and spotted from left to right (most to least concentrated). **(b)**. Growth curves of WT (blue), *pac1-ts* (red), S1 (grey) and S2 (yellow) at 37°C during +/- 64h in liquid medium. Error bars represent $CI_{95\%}$ over 2 biological replicates.

Based on those result, we decided not to work on the S1 strain anymore and to concentrate on the S2 strain that, for clarity, will be referred to as the *pac1-ts nda3 T216+T* strain for the rest of the manuscript. One hypothesis arised at this point, considering the fact that the *nda3* mutation is in the third intron of the gene. The mutation in *pac1-ts* could cause a defect in the splicing process of *nda3*, either directly or indirectly through Pac1 documented function in snRNA biogenesis (Nabavi and Nazar 2010a; Yague-Sanz et al. 2021). Such a splicing defect could be the cause of the growing defect observed at restrictive temperature (Figure 8b). The suppressive mutation in *nda3* intron could allow to bypass the putative splicing defect, and thus allow growth at restrictive temperature. However, this possibility is surprising as many other introns (nearly half of the fission genes have introns) should also be affected. Nevertheless, it is possible that a modest defect in splicing is not phenotypically relevant for most genes. To test that hypothesis, three new strains were created either by CRISPR/Cas9 mutagenesis method or by mating:

- Deletion of the entire third intron in *nda3* by CRISPR/Cas9 mutagenesis (*nda3 [Δintron3])*
- Deletion of the entire third intron of *nda3* in *pac1-ts* strain by mating (pac1-ts nda3 *[Δintron3])*
- Mutation T216+T in third intron of *nda3* by mating (nda3 T216+T)

## 5.3. Evaluation of the phenotypes of the strains

To test our hypothesis, we started by measuring the growth of the strains described above at restrictive temperature (37°C) and semi-restrictive temperature (32°C).

At 32°C, we observed that the final OD (in stationary phase) for all three strains carrying the *pac1-ts* mutation was higher than for the other strains (Figure 9a). Since cell shape and size are known to affect turbidity, we attributed the final OD difference to the characteristic longer cell size of strains with *pac1* deficiency (Iino et al. 1991). In order to correct for this effect and help focusing on the differences during exponential growth, each curve was normalized by division by its last data point (Figure 9b). This was not done for the curves at 37°C (Figure 9c) because the strains carrying the *pac1-ts* did not reach stationary phase.

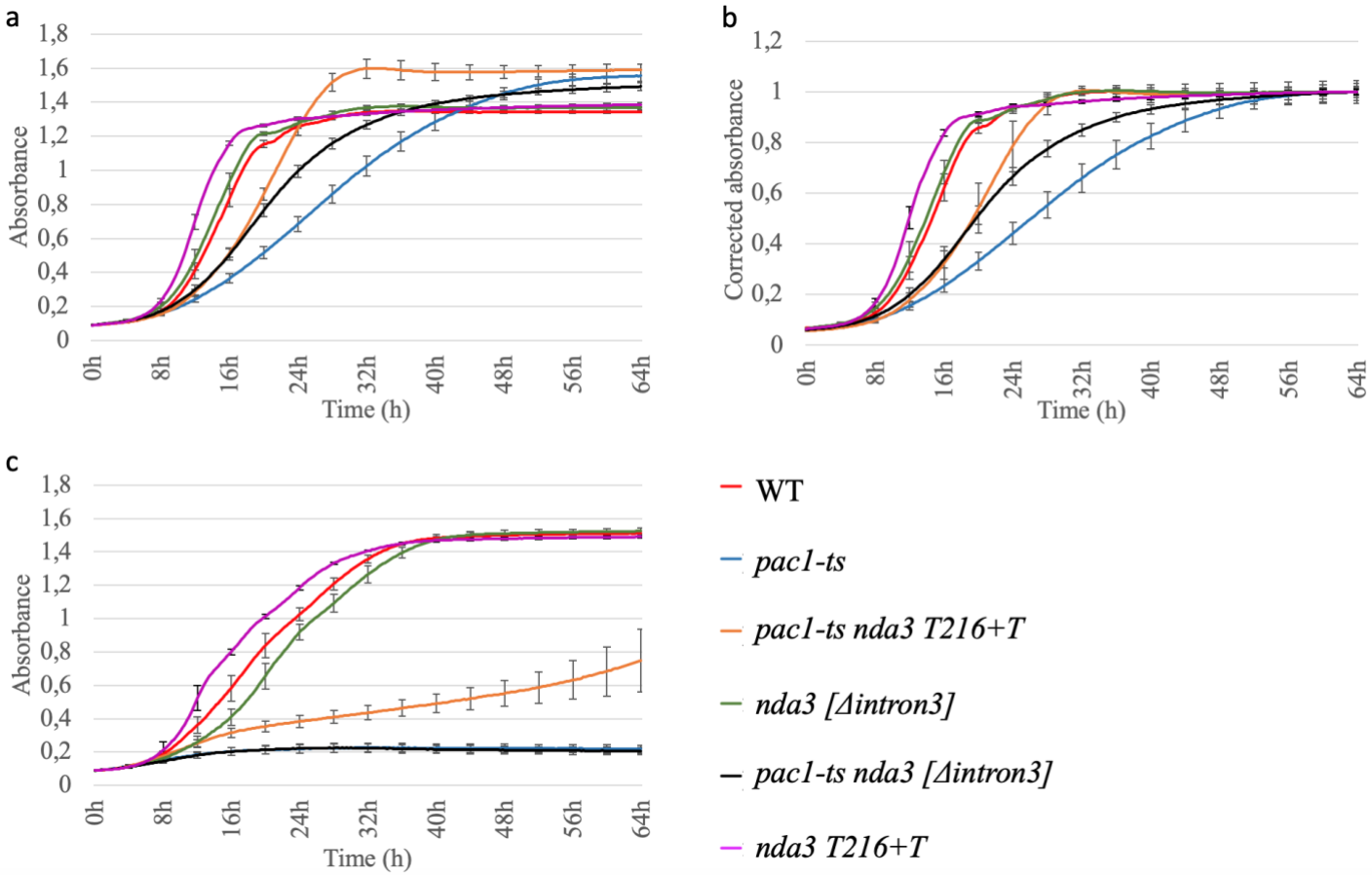



**Figure 9: Evaluation of the growth phenotype of the strains. (a).** Growth curves of WT (red), *pac1-ts* (blue), *pac1-ts nda3 T216+T* (orange), *nda3 [Δintron3]* (green), *pac1-ts nda3 [Δintron3]* (black), and *nda3 T216+T* (purple) at 32°C during 64h in YES medium. Error bars represent CI95% over 2 biological replicates. **(b).** Normalisation of the data at 32°C is done to correct for the differences observed in the plateau stage. Each data point in each curve is divided by the last data point in the corresponding curve. Error bars represent CI95% over 2 biological replicates. **(c).** The same as in **(a)** but at 37°C.

At the semi-restrictive temperature of 32°C (Figure 9a and 9b), the *pac1-ts strain* (blue) is able to grow but shows a marked growth defect. Interestingly, both the *pac1-ts nda3 T216+T* (orange) and *pac1-ts nda3 [Δintron3]* (black) are able to partially suppress the thermosensitive phenotype of the *pac1-ts*, as they both grow better, yet not as good as the WT strain. The single mutations *nda3 T216+T* (purple) and *nda3 [Δintron3]* (green) grow well, even better than the WT strain (red).

At 37°C (Figure 9c), the *pac1-ts* (blue) is not able to grow, as well as the *pac1-ts nda3 [Δintron3]* (black). In contract, the *pac1-ts nda3 T216+T* (orange) suppresses the

thermosensitive phenotype. The other three strains (WT, *nda3 T216+T*, *nda3 [Δintron3]*) grow well.

Together, these results only partially support our hypothesis that a splicing deficiency in the third *nda3* intron is a major determinant of the growth defect of *pac1-ts* strain. Indeed, while the deletion of that intron does not restore growth at 37°C in the *pac1-ts*, there is still a partial suppression at 32°C. Therefore, we aimed to further investigate the relationship between *pac1* and the splicing of *nda3*.

## 5.4. Intron retention and mRNA expression of *nda3* in *pac1-ts*.

According to our hypothesis, if the splicing of *nda3* is deficient when *pac1* is mutated, some non-spliced mRNA should accumulate in all the *pac1-ts* strains. This phenomenon could be responsible for the growing defect of these strains. Therefore, the expression level of the third intron of *nda3* was checked by RT-qPCR.

We measured the relative abundance of the third intron of *nda3* (*nda3 intron3* amplicon) in the WT, *pac1-ts*, *pac1-ts nda3 T216+T* and *nda3 [Δintron3]* strains (Figure 10. The *act1* housekeeping mRNA was used as a control, and the expression level of the second exon of the gene was also checked (*nda3* amplicon). The level of *mfs2* mRNA was used as a positive control.

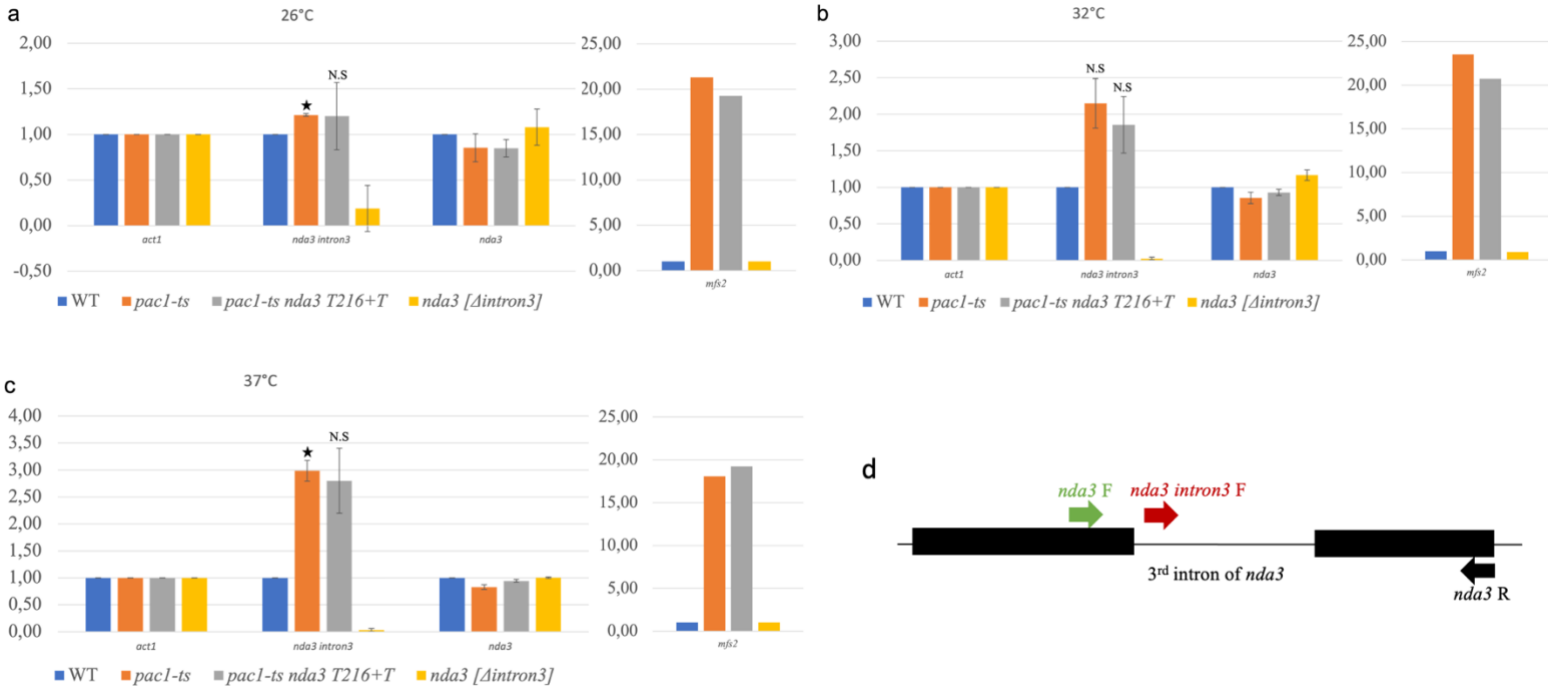



**Figure 10: Expression levels of *nda3* mRNA (*nda3*) or *nda3* unspliced intron 3 (*nda3 intron3*) in the indicated strains.** Statistically significant differences of mean are indicated by a (*) and were calculated with a Welch's t-test (unequal variances t-test) between the *pac1-ts* and the WT or between the suppressor (*pac1-ts nda3 T216+T)* and the *pac1-ts*. Error bars represent standard deviation over 2 biological replicates **(a).** strains were grown at 26°C. **(b)**. Strains were grown at 32°C. **(c)**. Strains were grown at 26°C, then shifted two hours 37°C. **(d)** Position of the oligonucleotides used for the qPCR on *nda3*. The *nda3 intron3* F oligonucleotide (red) is used with the *nda3* R (black) for the *nda3 intron3* to measure retention of *nda3* intron 3 (*nda3 intron3*). The *nda3* F (green) and the *nda3* R (black) are used to measure *nda3* mRNA level.

To further control that Pac1 activity is still deficient in the *pac1-ts* strain and the *pac1-ts nda3 T216+T* suppressor strain, we measured the relative abundance of the *mfs2* mRNA. *mfs2* is a protein-coding gene coding for a transmembrane protein of the major facilitator superfamily. It is known to be strongly repressed by Pac1 activity (Yague-Sanz et al. 2021). As expected, the mRNA is overexpressed in both strains carrying the *pac1-ts* allele, at every temperature, but remains low in the other conditions (Figure 10a, 10b, 10c). This indicates that the suppression of *pac1-ts* growth phenotype does not rely on a general compensation of Pac1 activity, an observation compatible with our working hypothesis.

We also note that the accumulation of *mfs2* in both *pac1-ts* strains is independent from the temperature, unlike the grow phenotype and the intron retention, which are temperature-dependent.

We also measured the relative expression of the third intron of *nda3* to control that it was properly deleted in the corresponding strain and to estimate the level of background in our qPCR assays. As observed on Figure 10, at every temperature, the level of the third intron of *nda3* in *nda3 [Δintron3]* is almost null, validating our strains. This result also underlines the high specificity of our qPCR assays.

The next step in the RT-qPCR was to evaluate the intron retention of the *pac1-ts* strain. Strikingly, we observed an important accumulation of the third intron of *nda3* in the *pac1-ts* compared to the wild-type, an accumulation that is dependent of the temperature: 1,1 time more at 26°C (p-value = 0,04281), about two-folds at 32°C (p-value = 0,1312) and about three-folds at 37°C (p-value = 0,04291).

Finally, the suppressive abilities of the *pac1-ts nda3 T216+T* strain was analyzed. Despite a small reduction, the intron retention observed in the *pac1-ts* strain does not seems to be suppressed (or at least the suppression is not statistically significant) by the presence of the additional mutation in the third intron of *nda3*.

The expression level of the second exon of the gene was also checked (*nda3* amplicon). Although there is no statistically significant difference, we observed a slight decreased expression at 32°C and 37°C in *pac1-ts* strain. This decreased expression is partially suppressed in the *pac1-ts nda3 T216+T* suppressor strain even if none of these differences are statistically significant.

## 5.5. Expression changes of β-tubulin level

The RT-qPCR experiments (Figure 10) partially confirmed the hypothesis stating that the *pac1-ts* mutation is responsible the accumulation of the third intron of *nda3*. The splicing process seems to be altered in this strain as the unspliced mRNA of *nda3* accumulates at restrictive temperature (Figure 10c and 10d). This accumulation of immature mRNA might have a consequence on the corresponding protein level. As *nda3* gene codes for β-tubulin, its expression level was analysed by Western Blotting in the WT, *pac1-ts*, *nda3 T216+T*, *nda3 [Δintron3]*, *pac1-ts nda3 T216+T* and *pac1-ts nda3 [Δintron3]* strains. If indeed the intron retention interferes with β-tubulin expression, we would expect to see a decrease in β-tubulin protein expression in the *pac1-ts* strain and possibly a rescue of this molecular phenotype in the other two *pac1-ts* strains (*pac1-ts nda3 T216+T* and *pac1-ts nda3 [Δintron3]* strains). The level

of β-tubulin in the *nda3 T216+T* and *nda3 [Δintron3]* is presumed to be unchanged compared to the WT.

For simplicity, and because we didn't have the time to test all temperatures, the expression level of β-tubulin was assessed at 32°C only (Figure 11), in the six strains previously mentioned. The expression level of α-tubulin is also checked to see if the potential variation of β-tubulin level echoed on the α-tubulin expression level, as both proteins work in a dimer conformation. A Cy5 labelling of total protein is used for normalization.

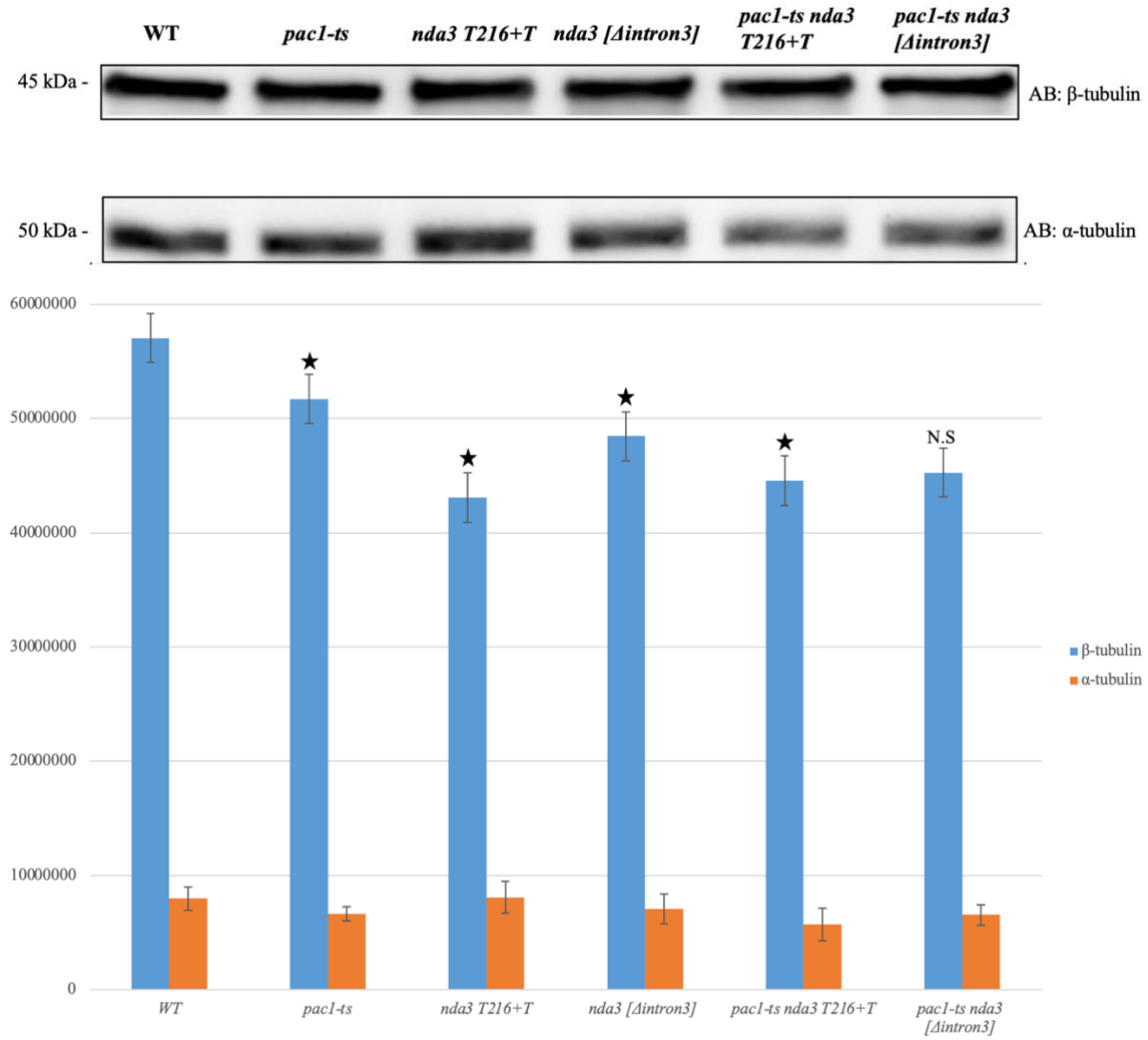



**Figure 11: β-tubulin and α-tubulin expression in the indicated strains grown at 32°C.** Normalization is made based on the signal of total proteins labelled with Cy5. Error bars represent CI95% over 2 biological replicates. Statistically significant differences of mean between the mutants and the WT are indicated by a (*) and were calculated with a Welch's t-test (unequal variances t-test).

As we can see on the Western Blot (Figure 11), the observed differences in the level of β-tubulin between the different strains are statistically significant but there is little variation in the level of β-tubulin. Moreover, no suppression of the decreased level of β-tubulin is observed between the *pac1-ts* strain and both *pac1-ts nda3 T216+T* and *pac1-ts nda3 [Δintron3]* strains. The observed differences in the level of α-tubulin are not statistically significant.

These Western Blots are not supporting our working hypothesis that proposes that a decrease in β-tubulin expression caused by intron retention explains the growth defect of *pac1-ts* and the suppression of the defect by a mutation in *nda3* intron. However, a possible explanation for that discrepancy is that the β-tubulin is sufficiently expressed in all the mutants but is not fully functional in *pac1*-ts. To test this hypothesis, the sensitivity of each strain to thiabendazole was tested.

## 5.6. Response to TBZ

Thiabendazole (TBZ) is benzimidazole fungicide that inhibits the grow of fungi by perturbing microtubule organisation, which ultimately blocks mitotic divisions. The mode of action of TBZ relies to its specific binding to β-tubulin, which inhibits microtubule polymerisation. Resistance to TBZ has been assessed when β-tubulin mutations caused an alteration in the binding affinity (Minagawa et al. 2021), but in general, other mutations in microtubule subunits or other perturbation in cytoskeleton organisation aggravate TBZ sensitivity at sub-lethal concentrations.

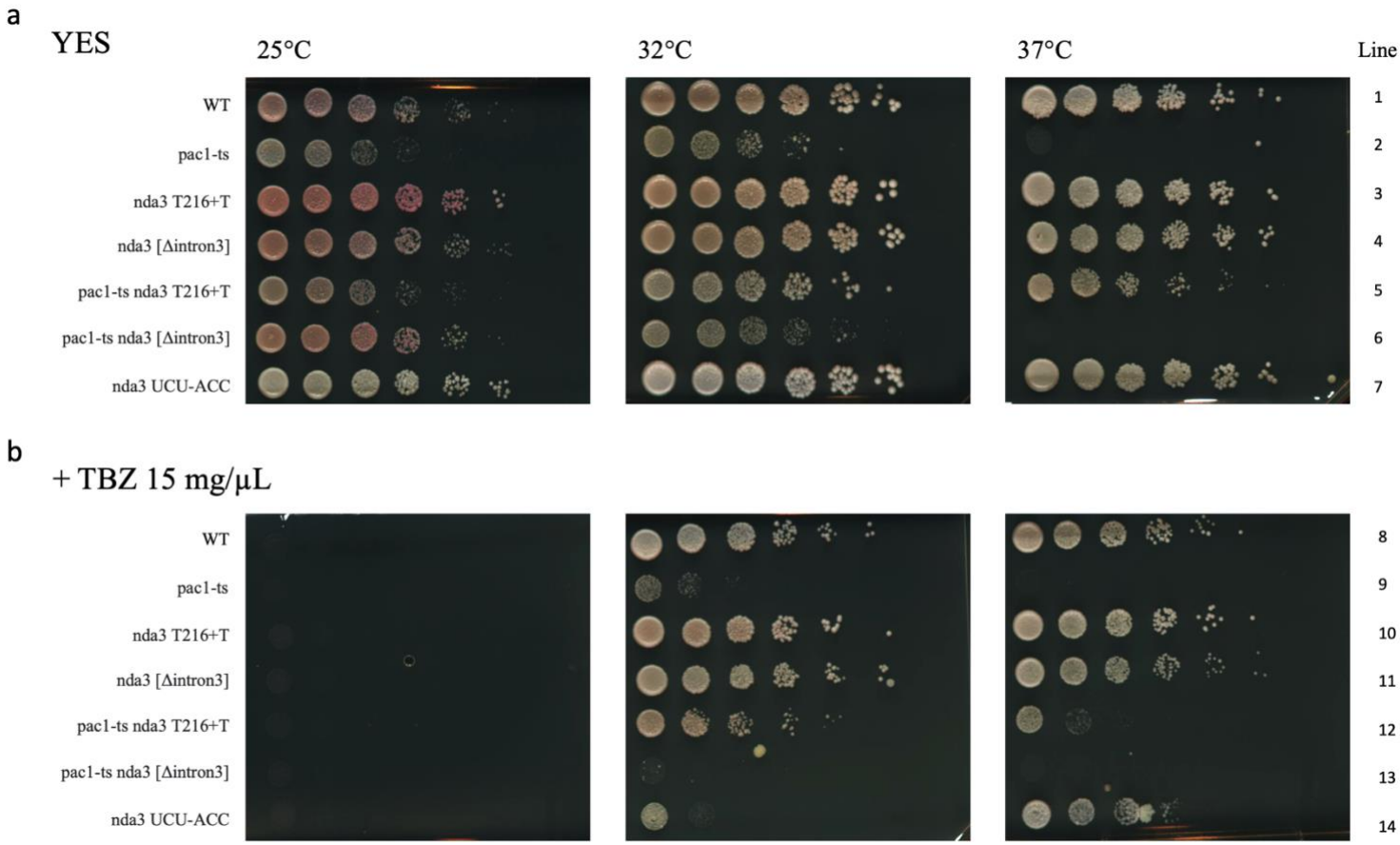



**Figure 12: pac1-ts sensitivity to TBZ**. Spot assays of WT, *pac1-ts*, *nda3 t216+T*, *nda3 [Δintron3]*, *pac1-ts nda3 T216+T*, *pac1-ts nda3 [Δintron3]*, and *nda3 UCU-ACC* at 25°C, 32°C and 37°C after 3 days either in YES medium **(a)** or in YES medium supplemented with 15 mg/mL of thiabendazole (TBZ) **(b)**. Cells were 5-fold diluted and spotted form left to right (most to least concentrated).

On the spot assays in the YES condition (Figure 12a), the growth pattern recapitulates what was previously observed in liquid culture in the Bioscreen experiments (Figure 9). Briefly, the

*pac1-ts* strain grow normally at 26° and has a slight growth defect at 32°C but its growth is stopped at 37°C compared to the growth of the WT strain (compare line 1 and line 2). The *nda3 T216+T* and *nda3 [Δintron3]* both grow normally at every temperature (lines 3 and 4). The *pac1-ts nda3 T216+T* strain shows a suppression of thermosensitive phenotype at 32°C, and even more strikingly at 37°C (line 5). The *pac1-ts nda3 [Δintron3]* strain also restores growth at 32°C but not at 37°C (line 6), which was seen in liquid medium.

In the YES supplemented with TBZ condition (Figure 12b), we can see that drug is not tolerated in any strain at 26°C. However, at 32°C, the WT, *nda3 T216+T* and *nda3 [Δintron3]* strains are only slightly affected by the presence of TBZ (compare line 7, 10 and 11). Their growth is comparable to what is seen without TBZ at the same temperature (Figure 12a). On the other hand, a hypersensitivity to TBZ is observed in the *pac1-ts* strain at 32°C (line 8). That hypersensitivity is suppressed in the *pac1-ts nda3 T216+T*, but somewhat aggravated in the *pac1-ts nda3 [Δintron3]* strain (compare line 8, 12 and 13). As a control for TBZ sensitivity, we also included a previously described TBZ-sensitive *nda3* mutant (*nda3 UCU-ACC*) that behaves as expected (line 7 and 14) (Finet et al. 2022).

The phenotypes at 37°C are similar to that at 32°C, although the effects are even more marked. Indeed, the growth of the *pac1-ts* and *pac1-ts nda3 [Δintron3]* strains is completely stopped (line 9 and 13). This phenotype is slightly suppressed in the *pac1-ts nda3 T216+T* strain (line 12). Both the *nda3 T216+T* and *nda3 [Δintron3]* strains grow normally at this temperature (line 10 and 11).

# 6. Conclusion and Perspectives

To explain the suppression of the *pac1-ts* growth defect by mutation in *nda3* intron, our initial hypothesis was that the *pac1 G1024A* mutation affects the splicing of the third intron of *nda3*. Such effect could be mediated by either direct interaction of Pac1 with the *nda3* mRNA, or by an indirect effect on the synthesis of components of the spliceosome. We currently do not have any data supporting the direct effect hypothesis. Regarding a possible indirect effect of Pac1 on *nda3* splicing, our favored hypothesis relies on a defect in snRNAs processing, which was previously described in the *pac1-ts* mutant (Nabavi and Nazar 2010a; Potashkin and Frendewey 1990; Yague-Sanz et al. 2021). Indeed, as components of the spliceosome, snRNAs have an important role in the splicing of pre-mRNAs (Lerner et al. 1980). Therefore, we could hypothesize that the *pac1-ts* mutation reduces the pool of functional snRNAs and causes a splicing defect, notably of the *nda3* mRNA. This putative splicing defect of *nda3*, combined with the snRNA alterations, may cause the growth defect observed in *pac1-ts*.

In support to this hypothesis, we observed a temperature-dependent increase in intron retention in the *pac1-ts* strain (Figure 9). This increased introns retention was confirmed with percent spliced-in (PSI, a measure of intron retention) analysis from RNA-seq data that shows an increase of 4-folds in the retention of the third *nda3* intron in the *pac1-ts* (PSI = 8%) compared to the WT (PSI = 2%) (Figure 13b). However, the PSI analysis revealed that the splicing defect is not restricted to the third *nda3* intron but is rather widespread with hundreds of introns affected (Figure 13a), including *nda3* first and fourth introns. That widespread effect on splicing may not be surprising given that a snRNA processing defect (as in the *pac1-ts*) is expected to globally affect splicing.

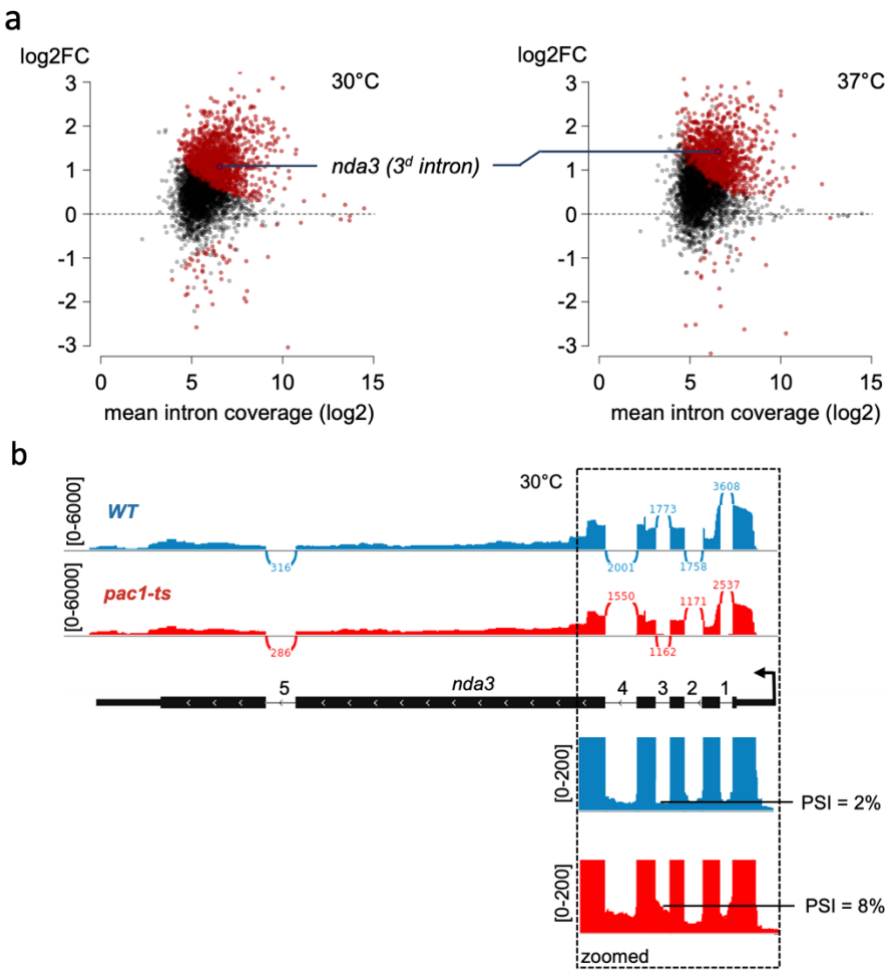



**Figure 13: Overall intron retention in *pac1-ts* mutant. (a)**. Log2 fold change in *pac1-ts* compared to WT grown at 30°C or shifted two hours at 37°C against mean expression for all *S. pombe* introns not overlapping any other exon (n=4232). The intronic read counts were normalized on their matched exonic read count prior to fold change calculation. Statistically significant (FDR-corrected p-value < 0.01) differences in intron retention are highlighted in red. **(b)**. Sashimi plot (read coverage highlighting the raw number of exon-exon splice junction reads) of the *nda3* gene, transcribed from right to left, in the WT and *pac1-ts* mutant at 37°C. On the zoomed-in bottom panel, the percentage spliced-in (PSI, the proportion of reads supporting inclusion against the reads supporting inclusion or exclusion) of the third *nda3* intron is indicated (C. Yague-Sanz, unpublished work).

Given the widespread effect of *pac1-ts* on splicing, it is somewhat surprising that a mutation in only one intron (*nda3 T216+T)* suppresses the growth defect (Figure 9b). To explain this discrepancy, we could hypothesize that intron retention is well tolerated for most genes, but not for *nda3*. Therefore, compensation of the splicing defect on *nda3* only would restore effective growth. This hypothesis takes support in previous finding in *S. cerevisiae* where the deletion of a single intron in α-tubulin was able to suppress the growth arrest phenotype of a splicing factor mutant (Burns et al. 2002). In their study, the splicing factor mutant also caused an overall splicing defect with intron retention causing a growth arrest, which is reminiscent to the *pac1-ts* phenotype. The α-tubulin intron deletion was able to rescue the phenotype, which indicates that the splicing defect was not deleterious for all the other introns but for this one. It should also be noted that *S. cerevisiae* has much less intron-containing genes that *S. pombe*. As *S. pombe* β-tubulin is also an essential protein, its correct splicing might be important as well in our system.

According to our hypothesis, we predicted that *nda3 T216+T* suppressive mutation would bypass the putative splicing problem and suppress the growth defect of the *pac1-ts* cells. This rise questions as to how an additional T in an intron could have this consequence on the phenotype of the strain. We know that the intron splicing is directed by the presence of three splicing signal. One of them, the PPT-3'ss, is a polypyrimidine tract located at the 3'end of the intron, between the BS and the 3'ss (Plaschka et al. 2019; Schwartz et al. 2008)(Figure 1a). It has been observed (Coolidge, Seely, and Patton 1997) that modifications of the polypyrimidine tract can affect the recognition of branch point sequences and even abolish the spliceosome assembly and thus the splicing. When adding some uridines to the polypyrimidine tract (Patterson and Guthrie 1991), the splicing of the gene is favoured as well as the correct utilization of the 5'ss. The *nda3 T216+T* is a mutation that actually extended the polypyrimidine track within *nda3* third intron (from 7 to 8 T), providing a molecular basis to explain the suppression.

To test whether our hypothesis was correct, we deleted the entire third *nda3* intron and assessed whether bypassing the splicing defect that way would suppress *pac1-ts* phenotype. Surprisingly, our results are not quite in accordance with that hypothesis. While the growth defect observed in the *pac1-ts* strain is suppressed by the *nda3 T216+T* mutation, the suppression is only partial with the full intron deletion (Figure 9). That discrepancy could be explained by the fact that protein implicated in the splicing of genes stay bound to the BS and 5'ss after the removal of the introns, and that they help increasing the export and the translation level of the mRNA, as both are linked (De Magistris 2021; Lei, Krebber, and Silver 2001). The removal of an intron might therefore cause unexpected consequences during the export and/or the translation of the mRNA beyond simply erasing a splicing defect.

In further disagreement with our hypothesis, the intron retention phenotype is not suppressed in the *pac1-ts nda3 T216+T* strain (Figure 10), indicating that the retention of the third intron of *nda3* is not directly responsible for the growth defect phenotype of *pac1-ts*. One explanation could be that the retention of the third intron of *nda3* have consequences on the splicing of the other introns of the gene. This splicing defect of the other *nda3*'s introns would then be responsible for the growth defect. The additional mutation in *nda3* would suppress the growth defect by restoring the splicing of the introns. Alternatively, a slight decrease in retention may be sufficient to partially suppress the growth defect and yet could go unnoticed by the current analyses' methods.

Finally, the differences in expression level of β-tubulin observed, although statistically significant, are small and no suppression is observed (Figure 11). However, *pac1-ts* strain exhibits a clear hypersensitivity to TBZ, which is suppressed in the *pac1-ts nda3 T216+T* strain but not in the *pac1-ts nda3 [Δintron3]* strain (Figure 12b). *pac1-ts* exhibits cytoskeleton defect, which is suppressed by the additional *nda3* mutation in third intron, but not by the deletion of the entire intron. β-tubulin function seems affected when *nda3* splicing is defective, in the *pac1-ts* strain.

We can speculate that the small effects observed on the mRNA and protein expression level might be sufficient to cause the growth defect observed and are in any case sufficient to have enabled the isolation of the suppressor during blind screening. In the end, the mechanism linking the T addition in the third intron of *nda3,* and the rescue of the grow caused by the *pac1-ts* mutation remains unclear.

The deletion of introns from three essential genes in *S. cerevisiae* (Juneau et al. 2006) show that they contribute positively to the fitness of the yeast. In fact, after the deletion of the introns, cells exhibit a growth defect, an increased sensitivity to drugs and a lowered expression of RNA. This is also consistent with our finding and correlates to our hypothesis stating that the splicing defect in *pac1-ts* is, at least in part, responsible for the growth defect of the strain. Removing introns from genes could also disrupt nucleosome binding (Juneau et al. 2006).

Many things are still to be done to understand the complex mechanism of the post-transcriptional regulation by Pac1. However, the next step would be to replace the third intron of *nda3* by an intron containing more consensus splicing signals, in the *pac1-ts* strain. In fact, the consensus signals found in *nda3* third intron are degenerated from the consensus signal of *S. pombe* (Figure 1). We know that the presence of consensus splice sites allow for the strain to have a better splicing efficiency (Schirman et al. 2021). If the strain with consensus signals in the third intron of *nda3* suppresses the thermosensitive phenotype of *pac1-ts*, we would be one step closer to demonstrate that the link between Pac1 and *nda3* goes through the splicing of the third intron of *nda3*.

Finally, the removal of all *nda3* intron would give us information on the effect of the third intron splicing on the splicing of the others. That could be done by CRISPR/Cas9 method by simply deleting the introns. Doing this, we would have an idea if the mechanism is intron dependent or not. Inserting the third intron of *nda3* in another gene would also inform us about the effect this intron has on the gene expression.

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