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Post-transcriptional regulation of sress-induced sexual differentiation by a ribonuclease

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POST-TRANSCRIPTIONAL REGULATION OF SRESS-INDUCED SEXUAL DIFFERENTIATION BY A RIBONUCLEASE

Mémoire présenté pour l'obtention

du grade académique de master 120 en biochimie et biologie moléculaire et cellulaire

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Post-transcriptional regulation of stress-induced sexual differentiation by a ribonuclease

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Summary

The switch between proliferation and sexual differentiation in fission yeast is tightly regulated by multiple regulatory pathways. This regulation relies on the perception of the environment, including the presence of pheromone of the opposite mating-type, stress or nutritional starvation. Among the negative regulators of sexual differentiation in *Schizosaccharomyces pombe*, the endoribonuclease Pac1 has previously been identified. Pac1 selectively targets RNAs possessing a stem-loop structure, promoting either their maturation or their degradation by exonucleases. Despite a well-established understanding of its molecular function, how Pac1 is regulated in the context of sexual differentiation is currently unknown.

This Master thesis aims to delve deeper into characterizing the role of Pac1, building upon prior laboratory findings indicating a rapid decrease in Pac1 abundance during nitrogen starvation. Firstly, through Western blot analyses, we were able to highlight Tor2, the catalytic subunit of the TORC1 complex, as one of the players governing Pac1 regulation. Further perturbation of the TORC1 pathway in condition of nitrogen starvation suggested a double regulation exerted on Pac1 during nitrogen stress. We also investigated the phosphorylation of two Pac1 residues, as a post-translational marker potentially involved in the stabilization of the protein. However, to date, we have not been unable to highlight a positive or negative role of those phosphorylation mark in Pac1 stability.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire

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Régulation post-transcriptionnelle de la différentiation sexuelle par une ribonucléase induite par un stress

GOSSELIN Elodie

Résumé

Le passage entre la prolifération et la différentiation sexuelle est étroitement régulé par de multiples voies de régulation. Cette régulation repose sur la perception de l'environnement, notamment la présence de phéromones, un stress ou la famine. Parmi les régulateurs négatifs de la différenciation sexuelle, l'endoribonucléase Pac1 a été identifiée récemment chez *Schizosaccharomyces pombe*. Pac1 cible sélectivement les ARN dotés d'une structure en tige-boucle, ce qui permet leur maturation ou entraîne leur dégradation par des exonucléases. Malgré une compréhension bien établie de sa fonction, les mécanismes en qui entrent en jeu en amont et en aval de Pac1 dans le contexte de la différentiation sexuelle sont actuellement inconnus.

Ce mémoire vise à approfondir la caractérisation du rôle de Pac1, en s'appuyant sur des résultats de laboratoire antérieurs indiquant une diminution rapide de l'abondance de Pac1 lors d'une privation d'azote. Premièrement, grâce à des analyses par Western blot, nous avons pu mettre en évidence Tor2, la sous-unité catalytique du complexe TORC1, comme l'un des acteurs gouvernant la régulation à la baisse de Pac1. La perturbation de la voie TORC1 en condition de privation d'azote suggère une double régulation exercée sur Pac1 durant un stress azoté. Dans un second temps, nous avons étudié la phosphorylation de deux résidus de Pac1, des marques post-traductionnelles potentiellement impliquées dans la stabilisation de la protéine. Cependant, jusqu'à présent, nous n'avons pas été en mesure de mettre en évidence un rôle positif ou négatif de ces marques de phosphorylation dans la stabilité de Pac1.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire

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Introduction

1. *Schizosaccharomyces pombe*

Schizosaccharomyces pombe, also known as fission yeast, is a unicellular organism widely researched in the field of cellular and molecular biology. Many mechanisms conserved in eukaryotes, in particular its cell cycle (Hoffman et al., 2015) are studied in this model organism.

S. pombe wild type is mainly found in haploid form and with a mating type. Yeasts are either "plus" or "minus" (denoted h+ and h-). This characteristic is genetically defined by the mating type locus, mat1, located on chromosome II. A homothallic strain (h90) incorporating the genetic information of both mating type and can change between them, as opposed to a heterothallic strain (Gutz & Doe, 1973; Hayles & Nurse, 2018; Yamamoto, 1996).

1.1. Life cycle

Under condition of nutritional abundance, *S. pombe* proliferates through mitosis, generating a haploid yeast population (Figure 1). This represents a form of asexual reproduction. During mitotic division, a septum divides the parent yeast cell in the center and produces two daughter cells of identical size (Hayles & Nurse, 2018; *Molecular Biology of the Fission Yeast*, 2012).

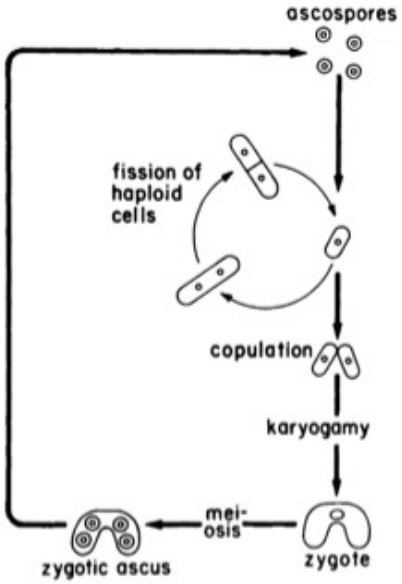

Figure 1. **Life cycle of Schizosaccharomyces pombe.** Through fission, haploid cells divide and proliferate. However, under environmental variations, cells can mate to form a zygote. Four spores are formed within an ascus and then released into the environment. Ultimately, these spores germinate and proceed miotic cycles to produce haploid cells (adapted from Molecular Biology of the Fission Yeast, 2012).

During significant stress and more particularly nitrogen starvation, *Schizosaccharomyces pombe* arrests its mitotic cell cycle in G1. Cells can enter a quiescent G0 stage or undergo sexual differentiation (Hayles & Nurse, 2018). At this point, two G1-arrested cells of opposite mating types can then mate and proceed to sporulation. Recognition between cells of opposite mating types depends on the secretion of pheromones and the exposure of pheromone receptors located on the membrane. Spores are the product of the meiosis of two haploid cells forming a diploid

unit. The four spores are grouped together in an ascus. Once the ascus membrane is ruptured, the spores are released and remain in latency. When environment becomes favorable for growth again, the spores germinate, enter vegetative division and proliferate in haploid form (Figure 1) (Hayles & Nurse, 2018; Petersen & Russell, 2016).

Sexual differentiation is notably based on successive transcriptional waves that allow mating, meiotic division and sporulation. Among these induced genes, *ste11* encodes a central transcription factor in sexual differentiation (Otsubo & Yamamoto, 2012). Indeed, a *ste11*-deleted mutant is sterile (Sugimoto et al., 1991). Ste11 is part of the HMG box protein family and can bind DNA at TR boxes. These TR boxes are found within the promoter of different key genes in sexual differentiation including *ste11* itself, which participates in a positive feed-back loop (Kunitomo et al., 2000; Sugimoto et al., 1991). The different genes upregulated by Ste11 include genes specific to the mating type leading to the expression of pheromones and pheromone receptors and genes specific to meiosis such as *mei2*. Being a key player in the commitment towards sexual differentiation, *ste11* is tightly regulated by at least four different pathways involving stress sensing, nitrogen availability, carbon availability, and pheromone sensing (Otsubo & Yamamoto, 2012).

These transcriptional waves result from the modulation of various signaling pathways, sensitive to the extracellular environment, allowing sexual differentiation under appropriate conditions. These signaling pathways include the mitogen-activated protein kinase (MAPK) pathway activated in the presence of pheromones, a second MAPK pathway triggered during environmental variations causing stress, the protein kinase A (PKA) pathway and the target of rapamycin (TOR) pathway.

2. The MAPK pathway induced by mating pheromones

As stated in the life cycle chapter, *S. pombe* has the capacity to carry out sexual differentiation under certain conditions, such as a modification of its environment triggering stress. However, in order to fuse, two yeasts must recognize each other (Petersen & Russell, 2016). For this purpose, *S. pombe* produces pheromones that are recognized by a mating partner of the opposite type. An h- mating yeast secretes an M-factor, a short 9 amino acids peptide, whereas an h+ yeast will, in turn, produces a 23 amino acids long P-factor. These pheromones are recognized on the surface by G-protein coupled receptors (GPCR). As a reminder, these are receptors composed of seven transmembrane domains, participating in numerous signaling pathways. Ligand recognition allows activation of coupled heterotrimeric G protein (Hilger et al., 2018). h+ and h- yeasts will present on their surface a Map3 M-factor receptor and Mam2 P-factor receptor respectively (Otsubo & Yamamoto, 2012; Sieber et al., 2023). As cited above, the expression of these proteins is up regulated by Ste11, initially activated by different signaling pathways described below (Otsubo & Yamamoto, 2012). Once the pheromones are bound at the level of their GPCR, this recognition induces the activation of the alpha subunit of the associated G protein, encoded by Gpa1. The activated alpha-GTP subunit coupled Gpa1 will stimulate a MAPK pathway (Figure 2) (Sieber et al., 2023). MAPK pathways are regulatory pathways allowing the transduction of an external signal into the body in order to have a response adapted to the type of stimulus felt (Kowalczyk et al., 2013). More precisely, it involves a cascade of phosphorylation events orchestrated by kinase. Activation of Gpa1 induces that of Byr2 (MAPKKK) leading to the phosphorylation of Byr1 (MAPKK), which in turn phosphorylates Spk1 (MAPK). This cascade will strengthen the expression of *ste11* and

other genes necessary for mating and meiosis. This is positive feedback at the level of the expression of *ste11*, reinforcing this process (Sieber et al., 2023).

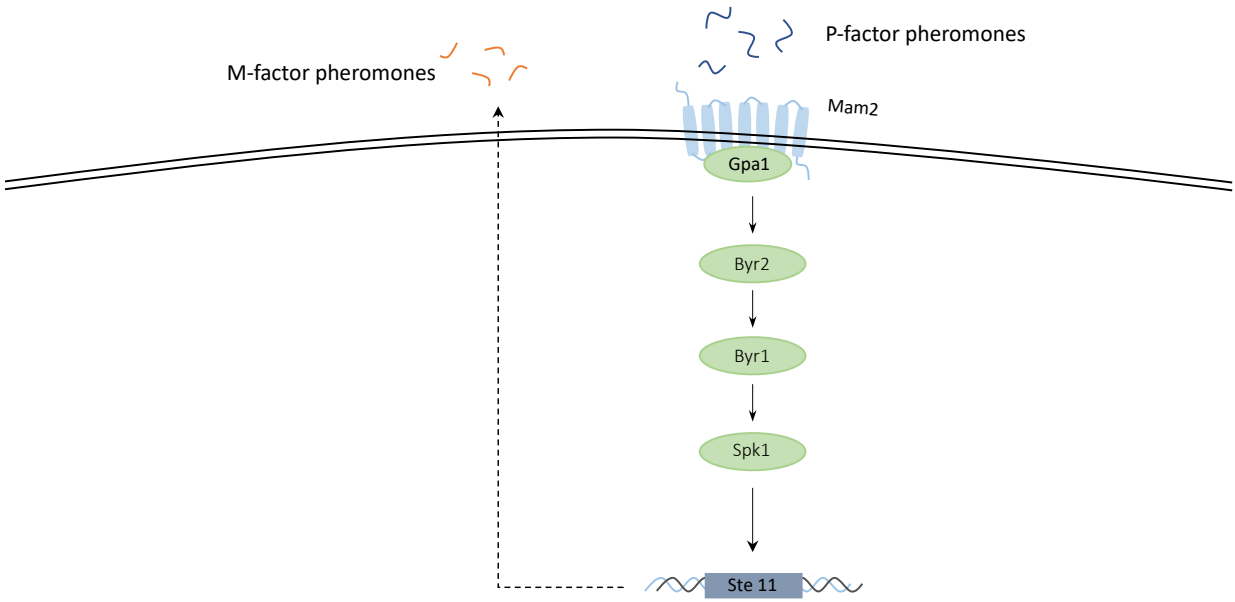

Figure 2. **The MAPK pathway induced by mating pheromones.** MAPK pathway (Byr2: MAPKKK; Byr1: MAPKK; Spk1: MAPK) activated following recognition of mating pheromones by their receptors. The expression of Ste11 positively regulates the expression of pheromones, which are then secreted into the environment. Normal arrows represent activation and dashed arrows represent undirect interaction (adapted from Otsubo and Yamamoto, 2012).

3. Stress-induced MAPK pathway

A wide range of environmental variations such as nutritional starvation, osmotic shock, oxidative stress, and heat exposure allows the induction of the mitogen-activated protein kinase (MAPK) pathway mediated by Sty1 (Figure 3) (Shiozaki & Russell, 1996). The stress response is initiated by Msc4, a response regulator that trigger a phosphorylation cascade leading to the phosphorylation of cytosolic Sty1, a MAPK. The activated form of MAPKKK, Wis4 and Win1 allows the phosphorylation and activation of Wis1, a MAPKK. Activated Wis1 phosphorylates Sty1, the MAPK (Shieh et al., 1998). The phosphorylated form of Sty1 translocates into the nucleus and accumulates there. Nuclear Sty1p phosphorylates Atf1 (Kar *et al.*, 2018). In the nuclear environment, Atf1 and Pcr1, both bZIP transcription factors can form a heterodimer, which has the ability to regulate target genes expression (Sánchez-Mir et al., 2020). A *wis1* or *sty1* deleted mutant in nitrogen starvation condition shows a reduced level of *ste11* transcription (Shiozaki & Russell, 1996). Nevertheless, the regulation of *ste11* by the MAPK/Sty1 pathway is mostly indirect as it relies on the transcriptional induction of *cgs2*, a gene coding for a phosphodiesterase. This phosphodiesterase negatively regulates a second pathway involved in the control of sexual differentiation: the PKA/cAMP pathway (Sánchez-Mir et al., 2020).

Activation of the Sty1 pathway and Atf1 leads to the expression of a phosphatase, Pyp2, known to be a negative regulator of Sty1 (Figure 3). Pyp2 dephosphorylates Sty1 at tyrosine 173 (Millar et al., 1995; Shiozaki et al., 1998). However, this same phosphatase is subject to degradation by the proteasome, which maintains Pyp2 at a low level (Kowalczyk et al., 2013).

A second phosphatase, Pyp1, can negatively regulate Sty1 in a manner independent of the Sty1 MAPK pathway. The abundance of Pyp1 is constant (Kowalczyk et al., 2013).

There is a second regulatory mechanism operated by Sty1 on *ste11*. Studies conducted in the laboratory have established a relationship between phosphorylation of RNA polymerase II and sexual differentiation (Coudreuse et al., 2010). RNA polymerase II notably governs the transcription of pre-messenger RNAs in eukaryotes. RNA pol II consists of 12 subunits, including Rhb1. Rhb1 contains the C-terminal domain (CTD) which is phosphorylated by cyclin-dependent kinases (CDKs). These phosphorylations allow the recruitment of actors necessary for the various stages of transcription (Schier & Taatjes, 2020). During nitrogen stress, activated Sty1 phosphorylates Lsk1, a CDK. Lsk1 would be recruited near *ste11* gene and enables the phosphorylation of the CTD. This process allows an upregulation of *ste11* transcription. Furthermore, a loss-of-function mutation of Lsk1 specifically impacts sexual differentiation through a reduction in *ste11* transcription (Figure 3) (Coudreuse et al., 2010; Sukegawa et al., 2011).

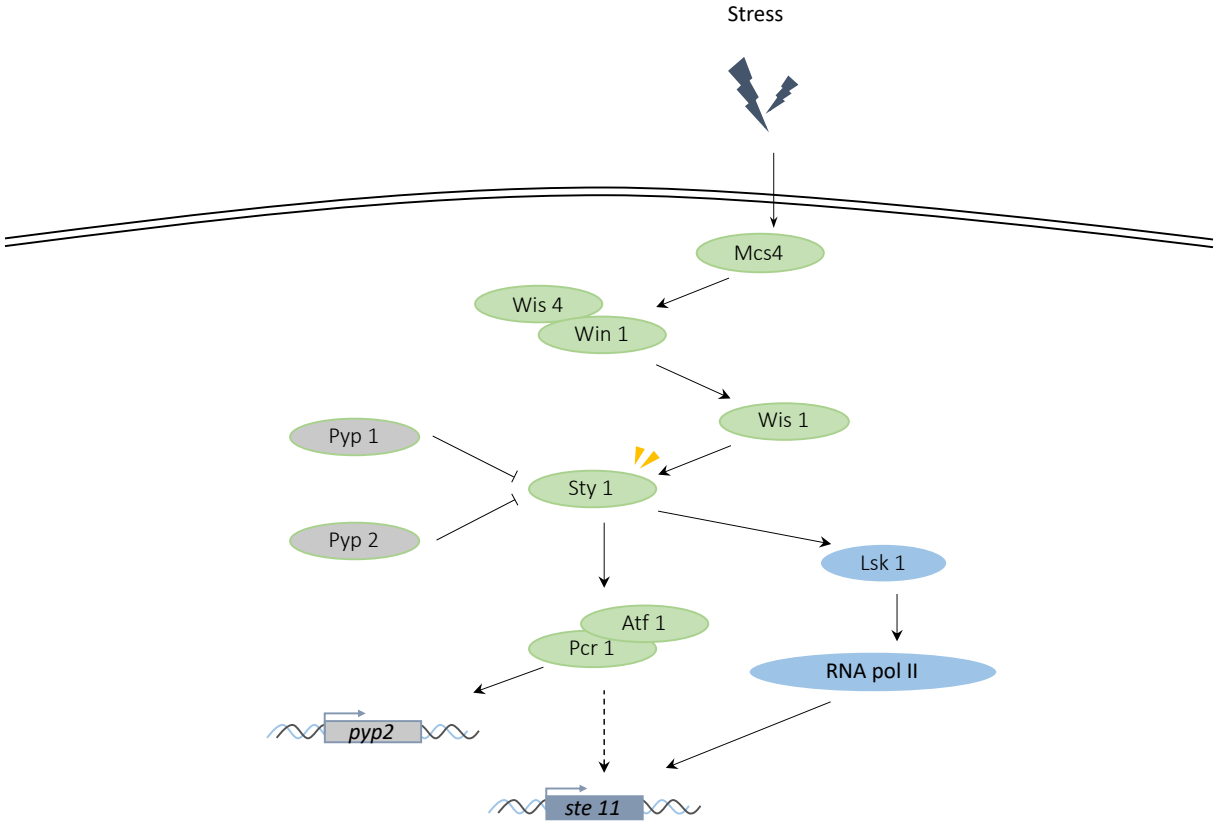

Figure 3. **Stress-induced MAPK pathway**. When the cell experiences stress, the signal transduction mediated by Mcs4, Wis4/Win1, and Wis1 leads to the activation of Sty1. Sty1 regulates the expression of *ste11* through Atf1/Pcr1 and Lsk1 and the expression of *pyp2*. Pyp2 and Pyp1 inhibit the activation of Sty1. Truncated arrows represent inhibition, normal arrows represent activation and dashed arrows represent undirect interaction (adapted from Millar et al., 1995; Otsubo & Yamamoto, 2012).

4. The cAMP/PKA pathway

Cyclic AMP (cAMP) is a crucial factor in mating whose concentration depends on the abundance of glucose and nitrogen in the extracellular environment (Hoffman, 2005; Isshiki et al., 1992; Sugimoto et al., 1991) . When nutrients – especially glucose – are low, the level of intracellular cAMP decreases and *S. pombe* enters sexual differentiation (Kunitomo et al., 2000; Mochizuki & Yamamoto, 1992).

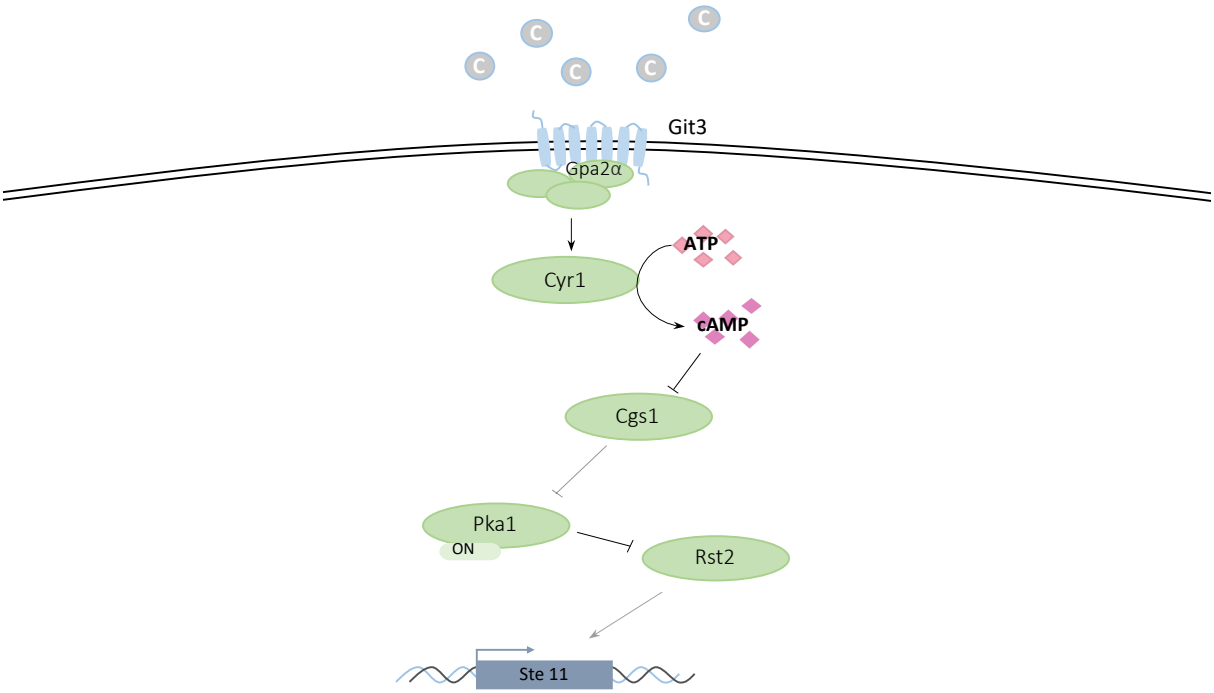

Figure 4. **The cAMP/PKA pathway**. In the presence of glucose, Cyr1 produces cAMP. By inhibiting Cgs1, presence of cAMP activates Pka1, which phosphorylates Rst2. The presence of glucose inhibits sexual differentiation. Truncated arrows represent inhibition, normal arrows represent activation and dashed arrows represent undirect interaction, circled C: carbon source (adapted from Otsubo and Yamamoto, 2012).

In a glucose-rich medium, *S. pombe* senses glucose in its extracellular environment through a membrane receptor, the seven-transmembrane protein Git3 (Figure 4). Upon glucose recognition by Git3, a heterotrimeric coupled G protein initiates the transduction pathway. This heterotrimeric G protein is composed of three subunits α (Gpa2), ß (Git5) and γ (Git11) (Hoffman, 2005; Otsubo & Yamamoto, 2012). Specifically, the alpha subunit (Gpa2A) is activated, and plays a crucial role in transmitting of the response. Gpa2a is encoded by the *gpa2* gene. An inactive mutant of *gpa2* allows the induction of sexual differentiation in a context of rich medium (Isshiki et al., 1992). In glucose-rich medium, Gpa2 activates the adenylate cyclase Cyr1 to synthesize cAMP. The high concentration of intracellular cAMP allows the binding of cAMP to the regulatory subunit of PKA, Cgs1. This blocks the inhibitory interaction between Cgs1 and Pka1, the catalytic subunit of PKA. Cgs1/cAMP interaction and the release of Pka1 allow the activity of PKA, which leads to the inhibition of sexual differentiation (Otsubo & Yamamoto, 2012). Negative regulation of genes by the cAMP/PKA pathway in high-glucose conditions involves the phosphorylation of Rst2, a zinc-finger transcription factor, by Pka1. When phosphorylated by PKA, Rst2 is sequestered in the cytoplasm and cannot activate the expression of *ste11* (Kunitomo et al., 2000). In contrast, in a situation of nutritional deficiency,

with a decrease in cAMP concentration, Cgs1 associates with Pka1. PKA being inactive, the non-phosphorylated form of Rst2 can induce the expression of important genes in sexual differentiation such as *ste11*, or of genes involved in glucose metabolism and gluconeogenesis (Ohtsuka et al., 2022).

5. Target of rapamycin pathway

Target of rapamycin (TOR) is serine/threonine kinase extremely conserved among eukaryotes. These proteins are part of the phosphatidylinositol kinase-related kinase family (Mak et al., 2021; Raught et al., 2001). TOR proteins were characterized in *Schizosaccharomyces cerevisiae*, in mutants resistant to rapamycin. Rapamycin, a macrolide, is a lipophilic molecule produced by *Streptomyces hygroscopicus*. By binding FKBP12, an intracellular receptor, rapamycin forms a complex that interacts with the rapamycin binding domain on TOR. This interaction inhibits the signaling cascade and inhibits cell proliferation. However, in *S. pombe,* vegetative growth is not affected by the molecule (Raught et al., 2001; Weisman, 2016). The mechanism operated by rapamycin is still not completely understood.

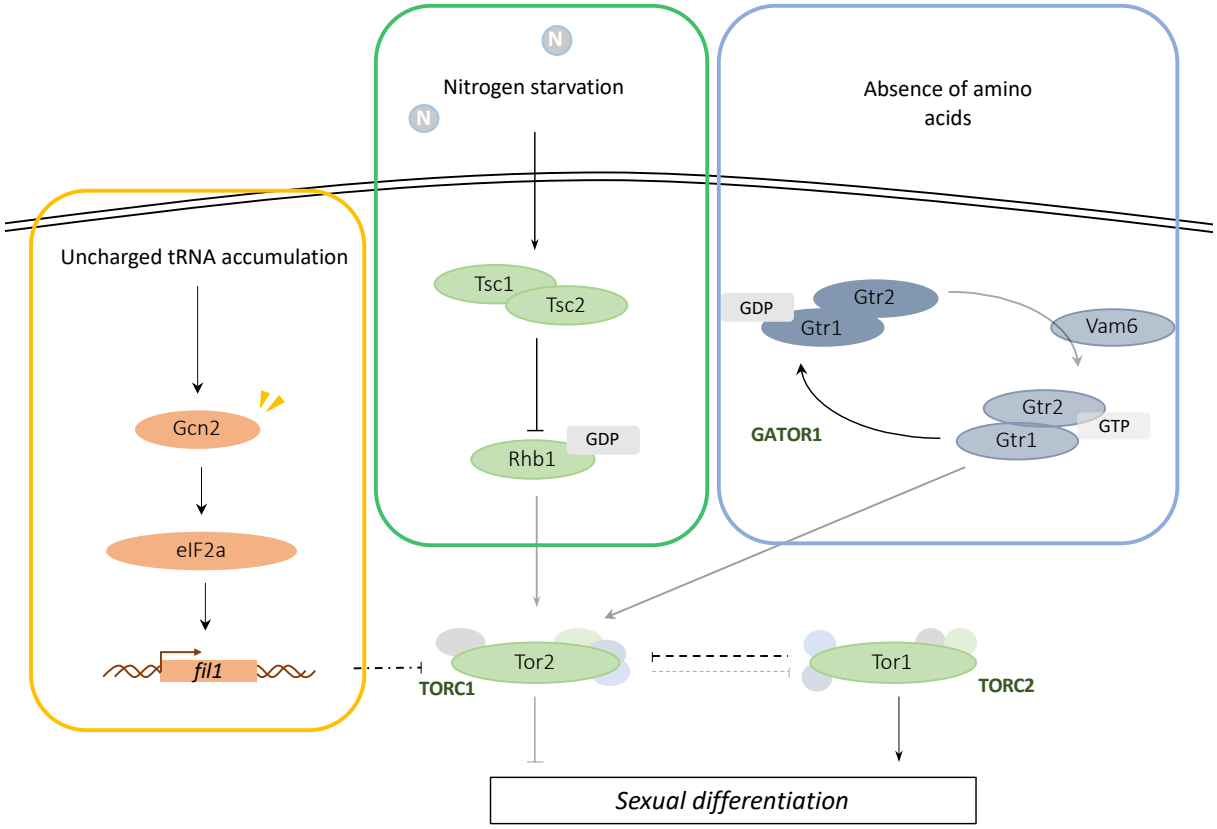

Figure 5. **Target of rapamycin pathway**. In the orange-framed section, Gcn2-mediated signaling responds to the accumulation of uncharged tRNAs and upregulates the expression of *fil1*. Fil1 inhibits the TOR complex 1. In the green-framed section, during nitrogen starvation, the Tsc1/Tsc2 complex inhibits Rhb1, leading to the inactivation of TORC1. In the blue-framed section, when the environment is deficient in amino acids, GATOR1 maintains the Gtr1-GDP/Gtr2 form, preventing the activation of TORC1. Truncated arrows represent inhibition, normal arrows represent activation and dashed arrows represent undirect interaction, circled N: nitrogen source (adapted from Fukuda et al., 2021; Otsubo & Yamamoto, 2012).

There are two target of Rapamycin complexes, named TORC1 and TORC2. In mammalians, the mammalian TOR (mTOR) kinase is present in both complexes, and the distinction is established by the subunits that compose them, In contrast, *S. pombe* has two homologs for the TOR kinase, Tor2 and Tor1, that respectively (and in a somewhat misleading manner) belong to the TORC1 and TORC2 complexes (Weisman, 2016).

These two complexes have opposite roles. Indeed, a *tor1* deletion mutant does not show an appropriate G1 arrest in nitrogen starvation and is unable to enter into sexual differentiation. Tor1 is crucial when the cell is confronted with stress such as nutritional stress (Álvarez & Moreno, 2006). On the contrary, a strain deficient in Tor2 activity stops its proliferation, enters prolonged G1 phase and shows an increase of *ste11* expression level (Matsuo et al., 2007). Cells overexpressing Tor2 are still able to respond to nitrogen deficiency and stop their cell cycle in G1, however they no longer have the capacity to proceed neither to mating or meiosis. (Álvarez & Moreno, 2006). *tor2* is an essential gene encoding a kinase that controls various process necessary for cell metabolism and more particularly in cell growth, such as protein synthesis, regulation of gene expression and regulation of permeases for uptake of amino acids, ribosome synthesis and inhibits autophagy. In addition, Tor2 is a negative regulator of sexual differentiation (Matsuo et al., 2007; Raught et al., 2001). Its role in differentiation is suggested through its interactions with Mei2, which is involved in this cell process (Álvarez & Moreno, 2006). Mei2 is phosphorylated by Tor2, this phosphorylation allows polyubiquitination of Mei2 leading to its degradation. In this manner, the induction of sexual differentiation is negatively controlled and the cell continues a mitotic cycle in vegetative growth (Otsubo et al., 2014). In summary, Tor2 (TORC1) promotes proliferation, whereas Tor1 (TORC2) promotes differentiation.

5.1. TORC1 regulation

Given that sexual differentiation is a highly regulated process. TORC1, an inhibitor of this phenomenon, is also subject to numerous regulations orchestrated by at least three different pathways: the Tsc1/Tsc2 complex that senses nitrogen availability, the GATOR pathway that responds to amino-acid availability, and Gcn2-mediated signaling that senses changes in the ratio between charged and uncharged tRNAs.

5.1.1. Tsc1/Tsc2 complex

The activation of TORC1 is notably mediated by Rhb1, a Ras GTPase (the Rheb homolog). The activity of Rhb1 is dependent on its GTP loading. When nitrogen is sufficiently abundant, Rhb1-GTP form interacts with TORC1 (Figure 5). However, during nitrogen starvation, the tuberous sclerosis complex (TSC) located upstream of Rhb1, is activated. The TSC consisting of Tsc1 and Tsc2, both GTPase-activating proteins (GAP). The active TSC complex inactivates Rhb1 through the hydrolysis of GTP bound to Rhb1. Consequently, TORC1 is no longer activated and loses its ability to inhibit sexual differentiation (Fukuda et al., 2021; Yang et al., 2017).

5.1.2. GATOR pathway

Additional GTPases are also involved in the regulation of TORC1. Indeed, Gtr1 and Gtr2 function as a heterodimer in the presence of extracellular amino acid and interact with TORC1 (Figure 5). The complex becomes active when Gtr1 is bound to a GTP, a process dependent on the presence of the amino acid and a GEF (GTP exchange factor) protein, Vam6

(Fukuda et al., 2021; Valbuena, Guan, et al., 2012). This interaction allows the activation of TORC1, maintains the yeast in vegetative division and blocks sexual differentiation. Mutants of Gtr1 and Gtr2 undergo sexual differentiation despite the presence of amino acids (Valbuena, Guan, et al., 2012). Conversely, in the absence of amino acid, TORC1 repression can occur independently of the TSC/Rhb1 pathway. The GATOR complex (GAP activity toward the Rag GTPases1) exerts GTPase-activating protein (GAP) activity on Gtr1. The Gtr1 form bound to a GDP loses its activating regulation on TORC1 (Chia et al., 2017).

5.1.3. Gcn2-mediated signaling

As mentioned above, Tor is a highly conserved kinase that connects the extracellular presence of nutrients such as nitrogen (a crucial component of amino acids) to cell metabolism. In *S. pombe*, a second conserved kinase fulfills a similar role: Gcn2. This kinase has been described in *Schizosaccharomyces cerevisiae* (Yuan et al., 2017). Gcn2 is a serine threonine kinase that senses amino acid deficiency. This deficiency is interpreted through an accumulation of uncharged tRNAs (=tRNAs not bound to amino acid) (Figure 5). Uncharged tRNAs bind Gcn2, inducing a conformational change allowing activation of the protein. The target of Gcn2 is eIF2α, the eukaryotic translation initiation factor. Phosphorylation of this factor generally slows down translation, but positively regulates the expression of transcription factors (Anda et al., 2017; Yuan et al., 2017). These transcription factors such as Fil1 in *S. pombe* are essential for the nitrogen stress response (Fukuda et al., 2021). Fukuda *et al*., suggests that activation of Gcn2 in response to amino acid deficiency would induce autophagy by inhibition of TORC1 through indirect regulation. This hypothesis is supported by the fact that this mechanism is already present in *S. cerevisiae* and mammals.

The relationship between Gcn2 and TORC1 is characterized at different scales. TORC1 has an inhibitory role on Gcn2. When TORC1 is active, it promotes translation directly, but also by inhibiting Gcn2 (Valbuena, Rozalén, et al., 2012). Fukuda *et al*., propose that in a nitrogen-free environment, TORC1 is inactive through the GATOR and Tsc1/Tsc2 pathways and that Gcn2 reinforce this inactivation.

6. Pat1

However, in addition to the different pathways described above, sexual differentiation is regulated by other factors, including Pat1. *pat1* encodes a serine threonine kinase which is a negative regulator of meiosis, preventing *S. pombe* cells from entering it from the haploid state. A *pat1*-defective mutant conjugates and sporulates despite a rich growth medium (Nurse, 1985). More precisely, Pat1 was characterized in a *pat1*-defective mutant strain which exhibited anarchic meiosis (Iino et al., 1991).

Pat1 notably has a direct role on the key transcription factor for sexual differentiation, Ste11 (Yang et al., 2017). Pat1 phosphorylates Ste11, this phosphorylation allows the binding of a 14-3-3 protein, Rad24. The interaction between phosphorylated form of Ste11 and Rad24 sequesters the transcription factor and stops the accumulation of Ste11 in the nucleus, and ultimately its own amplification (Otsubo & Yamamoto, 2012). Meiosis is a very controlled process and is notably dependent on the RNA binding protein Mei2 encoded by *mei2*. Its role is preponderant and intervenes early in the division process (Otsubo et al., 2017). This regulatory protein is one of the targets of Pat1. Pat1 phosphorylates Mei2 (Yamamoto, 1996). As discussed above, Mei2 is also a target of TORC1, the resulting phosphorylation of Pat1 and

TORC1 leads Mei2 to the pathway for proteasomal degradation following ubiquitination. This inhibition blocks the yeast in a mitotic cycle. During a starvation, Mei2 is only phosphorylated by Pat1, due to the inactivation of TORC1, this allows the induction of mating. Finally, when Mei2 is no longer phosphorylated at all, the protein allows the initiation of meiosis (Otsubo et al., 2014). Meiotic division is lost upon loss of function of Mei2 in a *pat1* mutant (Yamamoto, 1996).

7. Pac1

In order to discover a new negative regulator of meiosis, Watanabe and colleagues screened for genes whose overexpression could suppress the untimely meiosis occurring in a *pat1*-deficient strain (Watanabe et al., 1988). This is how Pac1 (Pat1 compensation) was first discovered. The function of Pac1 was first inferred based on homology with RNases III present in *Escherichia coli* (Iino et al., 1991; Xu et al., 1990). RNAses are ribonucleases involved post-transcriptionally in the maturation and degradation of RNAs. More particularly, RNAse III are endoribonucleases, targeting double stranded RNAs which in the case of Pac1 adopt typical stem-loop structures. In some cases, Pac1-dependent RNA cleavage allows the degradation of extremities by 3' -5' and 5'-3'exonucleases, leading to transcription termination (Figure 6B) (Yague-Sanz et al., 2021).

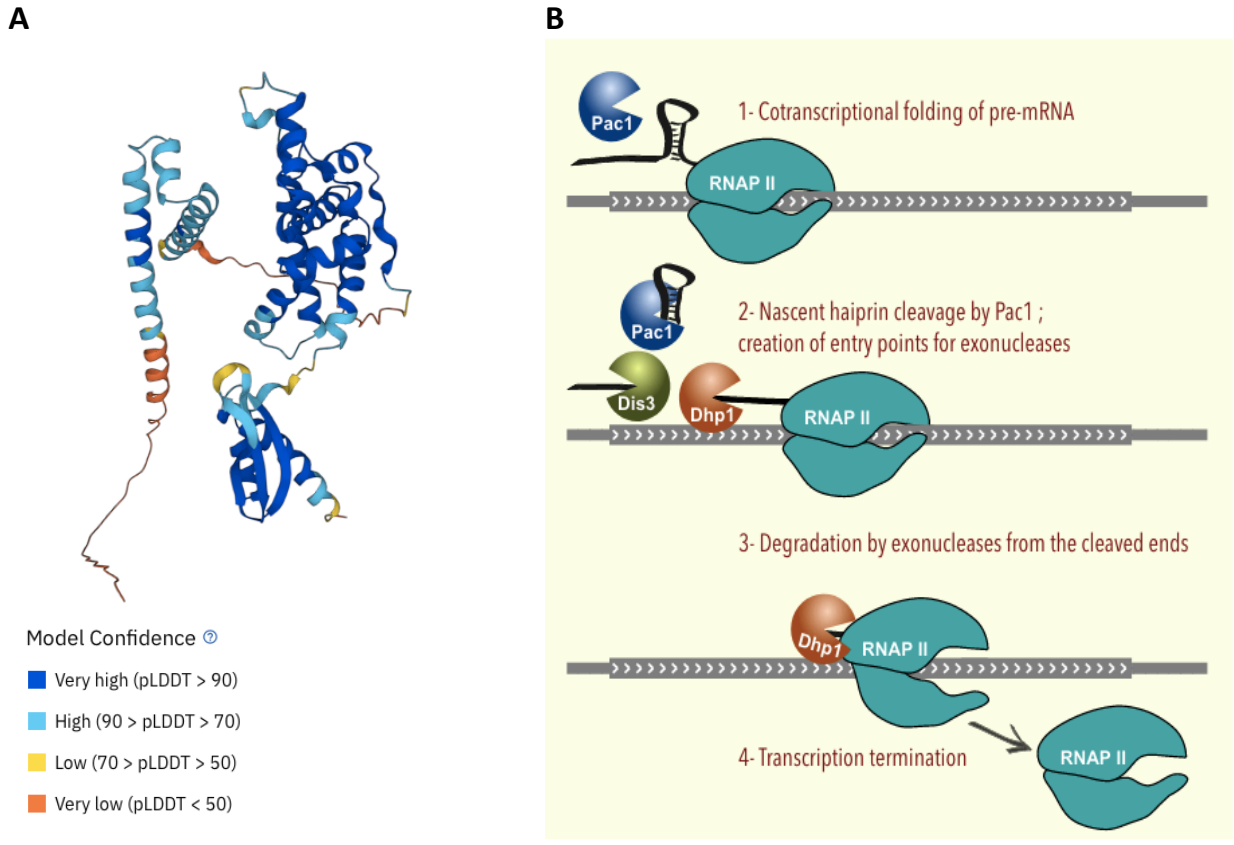

Figure 6. **Structure and function of Pac1**. **A**. Predicted structure of Pac1 on the AlphaFold Website, with the legend indicating the confidence level of the model. **B**. Pac1 cleaves at the hairpin structure of the pre-mRNA. This endonucleolytic cleavage facilitates access to exonucleases. Degradation occurs in both directions, from 3'-5' and from 5'-3', leading to transcription termination (Yague-Sanz et al., 2021).

Pac1 is an essential protein composed of 363 amino acids (Figure 6A) (Iino et al., 1991; Xu et al., 1990). It is composed of the two predicted domains, the Ribonuclease III domain is located between amino acids 139-262 and an RNA binding domain at 285-356 amino acids (Prosite Website) (Rotondo et al., 1997). The N-terminal domain (1-139) might contain a homodimerization domain. This prediction is based on the comparison with Pac1 homolog Rnt1 in *S. cerevisiae* whose N-terminal domain serves for the homodimerization of the protein (Lamontagne et al., 2000). Dominant negative phenotypes of Pac1-deficient strains further supports the idea that Pac1 also forms heterodimers in *S. pombe* (Zhou et al., 1999).

However, the link between Pac1 molecular function and its biological role opposing sexual differentiation is still not known. Nevertheless, different hypotheses have been proposed, for instance a potential involvement of Pac1 in the degradation of RNAs crucial for meiosis and in sexual differentiation (Iino et al., 1991).

Objectives

In order to better understand the role of Pac1 in the repression of sexual differentiation, we studied the expression of Pac1 in the early stages of nitrogen starvation or in the presence of a poor nitrogen source. Preliminary data from the lab showed that the level of Pac1 decreases dramatically over three hours of nitrogen starvation (V. Migeot & C. Yague-Sanz, unpublished). After confirming this observation, we investigated how the Pac1 down-regulation is regulated by implementing perturbations in the various signaling pathways involved in sexual differentiation and using the down-regulation of Pac1 as a read-out. Furthermore, a recent proteomic study has revealed two potentially phosphorylatable amino acids in the Pac1 sequence (Mak et al., 2021). We hypothesize that this phosphorylation could be implicated in the down-regulation of Pac1. Accordingly, we tried to interfere with the phosphorylation of these residues to observe the impact on the abundance of Pac1.

Results

1. Pac1 decreases over time under nitrogen starvation

In order to validate preliminary data, we performed a western blot of the wild-type strain of this study. Analysis of the abundance of a TAP-tagged version of Pac1 during three hours of nitrogen starvation reveals that Pac1 decreases over time and becomes undetectable after three hours in nitrogen-free medium (EMM-N) (Figure 7A, B). The blot reveals two bands for Pac1-TAP, both presenting a dramatic decrease in intensity over the starvation (Figure 7A).

To assess the sensitivity of the regulation carried out on Pac1, we tested the impact of other nitrogen stresses. Indeed, the cells respond not only to the presence/absence of nitrogen in the medium, but also to the quality of the nitrogen source with bad nitrogen sources triggering a TOR-dependent stress response (Petersen & Russell, 2016). Traditionally the minimal medium used (EMM) contains 20mM ammonium chloride, which is considered as a good nitrogen source. However, this nitrogen source can be substituted with 20mM glutamic acid, which is also a good nitrogen source although it requires different transporters for its entry into the cell (Petersen & Russell, 2016). In contrast, proline is bad nitrogen source, and even though it is sufficient to sustain growth and do not fully trigger sexual differentiation (Petersen & Russell, 2016). Here we tested a shift between EMMG, containing glutamic acid (good nitrogen source) to EMMP, containing proline (bad nitrogen source).

The western blot resulting from this shift from glutamate to proline reveals that Pac1 also decreases over time, although the decrease is somewhat slower compared to an EMM-N shift (Figure 7B). We also tested a shift from minimal medium with 20 mM ammonium chloride to minimal medium with 20 mM proline. Again, Pac1 decreased in abundance over time (Figure 7C). Examination of these Western blots indicates that the reduction in the quality of the nitrogen source also down-regulates Pac1.

Additionally, we used cyanine 5 to label all the proteins in our samples (see material and methods). Protein degradation is often observed over the course of the nitrogen starvation, which we attribute to increased autophagy and increased expression of cellular protease following as a response to the nitrogen stress (Corral-Ramos et al., 2022; Weisman, 2016). Importantly, we observe a reduction in degradation of total proteins when shifting the medium to EMMP compared to EMM-N, which makes the reduction in Pac1 expression easier to interpret. For the following experiments, we stressed the cells with either EMM-N (for the most ancient experiments) or EMMP (for the most recent ones, since this is now the preferred method) (Figure 7C). Note that uncropped blots for all experiments showing both Pac1 and total proteins are presented in the Annexes. Also, another way to reduce protein degradation is discussed in the perspectives.

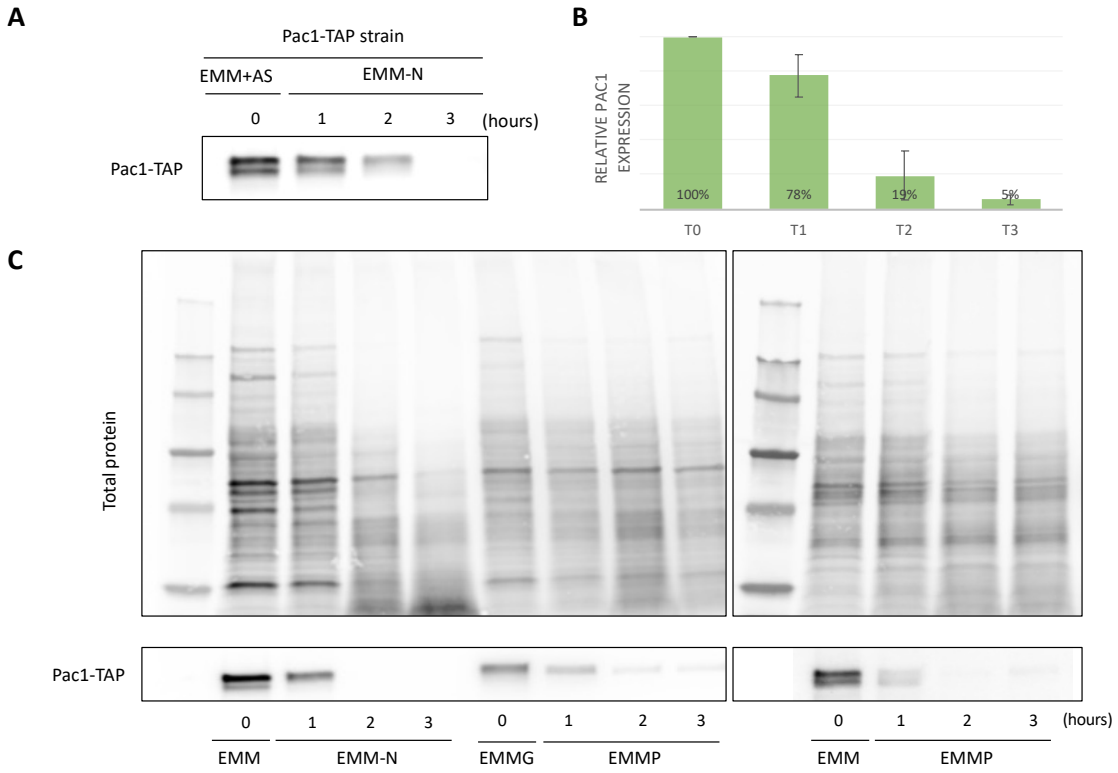

Figure 7. **Pac1 decreases under nitrogen starvation**. **A**. Representative western blot analysis of the level of TAP-tagged Pac1 over a period of 3 hours under nitrogen starvation (EMM-N) in a wild-type strain. **B**. Quantification of the western blots in (A). Error bars correspond to standard deviations. (n = 4 biological replicates). **C**. Representative western blot analysis of the level of TAP-tagged Pac1 over a period of 3 hours under nitrogen starvation (EMM-N) or in presence of a poor nitrogen source (EMMP) in a wild-type strain.

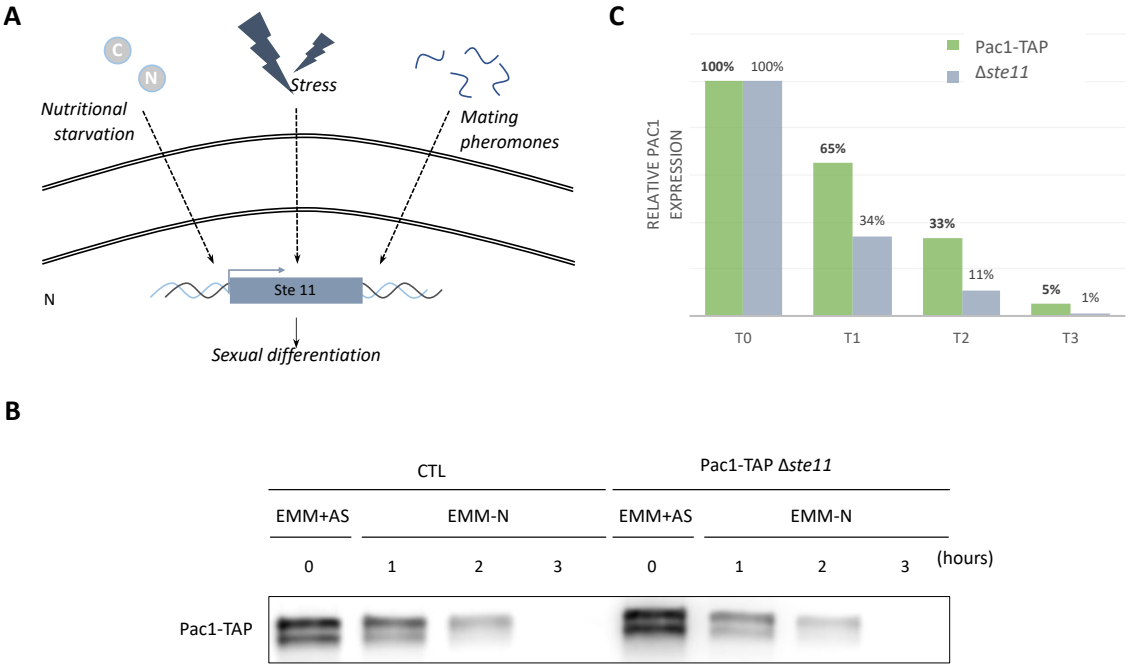

Figure 8. **Ste11 is not involved in the regulation of Pac1. A**. Pathways inducing transcription of *ste11* in the context of sexual differentiation (adapted from Otsubo & Yamamoto, 2012). Dashed arrows represent indirect activation. N: nucleus, circled N: nitrogen source, circled C: carbon source. **B**. Western blot analysis of TAP-tagged Pac1 over three hours of nitrogen starvation (EMM-N) in wild-type and Δ*ste11* strain. **C.** Quantification of the western blot in (B) (n= 1).

2. The regulation of Pac1 is independent of the Ste11 transcription factor

To understand how Pac1 is regulated during nitrogen depletion, we chose to target a critical factor in sexual differentiation, Ste11. Indeed, the transcription of *ste11* is up-regulated by multiple pathways that can be activated during nitrogen deficiency and Ste11 itself controls the expression of many genes involved in sexual differentiation and meiosis (Figure 8A) (Otsubo & Yamamoto, 2012). The western blot resulting from the nitrogen-free medium shift of the *ste11* deleted strain shows a decrease in TAP-tagged Pac1 over time. This decrease is comparable to the control of the experiment (Figure 8B, C). Based on the result we can conclude that the down-regulation of Pac1 in nitrogen starvation is independent of Ste11. Therefore, we can exclude sexual differentiation actors downstream of the Ste11 transcription factor and focus on the upstream factors. These include the MAPK pathway mediated by Sty1, the protein kinase A (PKA) pathway and the target of rapamycin (TOR) pathway, three signaling pathway that relay extracellular signals to promote *ste11* transcription and sexual differentiation.

3. Disruption of the cAMP/PKA pathway does not impact the level of abundance of Pac1

During nitrogen starvation, the level of intracellular cAMP decreases allowing cells to enter sexual differentiation (Sugimoto et al., 1991) (Figure 9A). To counteract this pathway, we added high concentration of extracellular cAMP in the medium to mimic an abundance of nutrients and promote vegetative growth (Laboucarié et al., 2017; Watanabe et al., 1988). Western blot analysis and quantification of the condition in which we added 5 mM cAMP to the nitrogen-free medium show a decrease in the level of abundance of the Pac1-TAP tagged protein over time, similar to the unsupplemented control medium (Figure 9B, C). To confirm that Pac1 down-regulation does not depend on cAMP, we used glucose starvation as a way to increase intracellular cAMP independently from nitrogen starvation. In glucose starvation, we do not observe a decrease in the abundance of Pac1. On the contrary, we even observe an increase of Pac1 over time, with some fluctuation between replicates (Figure 9D, E). Together our results indicate that the level of Pac1 is not impacted by positive or negative modulations of the cAMP/PKA pathway. In addition, we note that the lower band of the Pac1 doublet observed in the western blot analysis of cells cultured in a rich medium (YES) is weaker, or even absent in these conditions, compared to what is observed in a minimal medium (EMM) culture condition (Figure 9B, D).

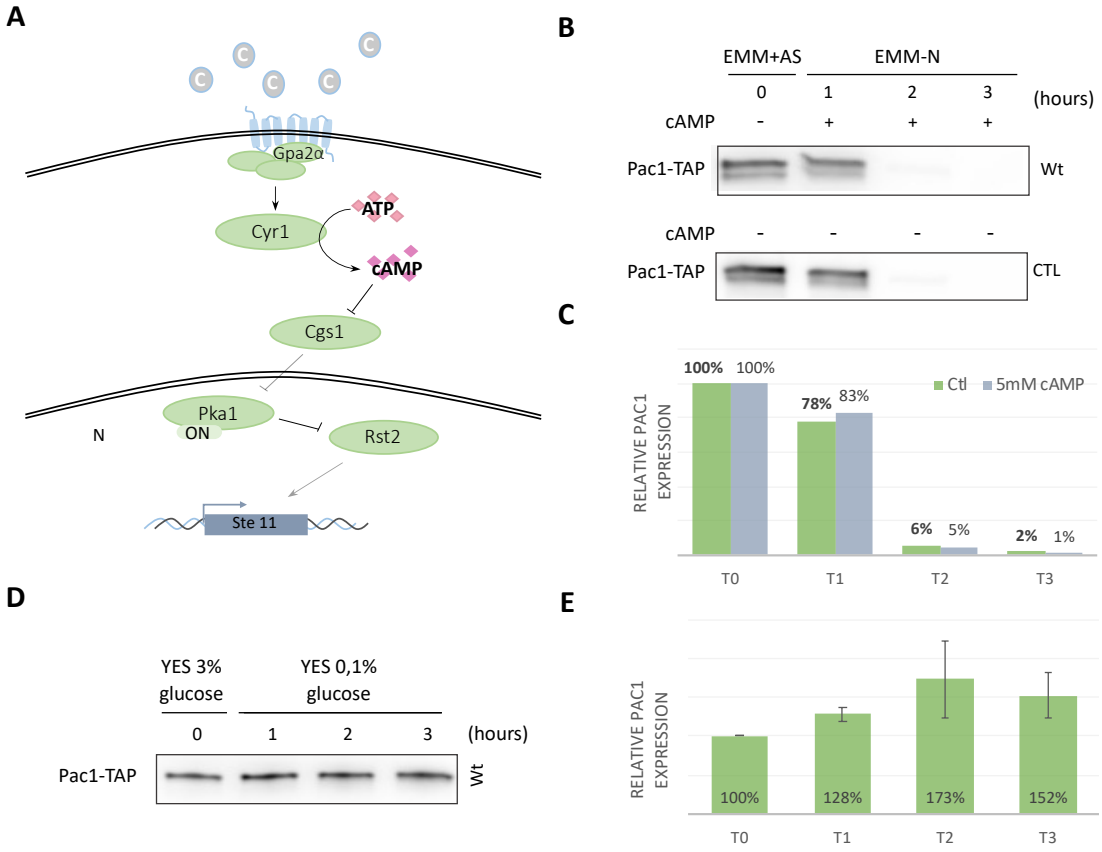

Figure 9. **The PKA/cAMP pathway is not required for the regulation of Pac1. A.** PKA pathway induced in the presence of glucose (adapted from Otsubo & Yamamoto, 2012). Truncated arrows represent inhibition, normal arrows represent activation, N: nucleus, circled C: carbon source. **B.** Western blot analysis of TAP-tagged Pac1 over three hours in nitrogen-free medium (EMM-N) supplemented or not with 5 mM cAMP. **C.** Quantification of the western blot in (B) (n= 1). **D.** Western blot analysis of TAP-tagged Pac1 over three hours of glucose starvation (YES 0.1% glucose) in a wild-type strain **E.** Quantification of the western blot in (D) (n = 3 biological replicates).

4. The Sty1 pathway does not regulate Pac1

The Sty1-mediated MAPK pathway is also activated during nitrogen starvation (Shiozaki & Russell, 1996) and is therefore another potential regulator of Pac1 expression (Figure 10A). To test whether the Sty1 protein has a role in the regulation of Pac1, we used a previously characterized analogue-sensitive (AS) version of Sty1 (Zuin et al., 2010). AS-kinases are mutated in their catalytic pocket to render them specifically sensitive to bulky ATP analogues (1NM-PP1 in this case). Similarly, to the control treated with the same concentration of 1NM-PP1, we observe for the Sty1 analogue-sensitive strain a decrease in Pac1 abundance in nitrogen starvation (Figure 10B, C). To confirm these data suggesting that Pac1 level is independent of the Sty1 cascade, we added sorbitol to the culture medium at a concentration of 1M, which causes osmotic stress and induces the MAPK/Sty1 pathway (Chen et al., 2003). Western blot quantification shows a slight decrease in the proportion of TAP-tagged Pac1 one hour after the addition of 1M sorbitol, followed by a continuous increase over time (Figure 10D, E). The pattern of Pac1 expression during osmotic stress being very different than in nitrogen starvation, we conclude that Pac1 regulation is nitrogen starvation is not mediated by the MAPK/Sty1

pathway. From these experiments, we note once again that a Pac1 doublet is visible in minimal medium (Figure 10B), whereas only one band is visible in rich medium (Figure 10D).

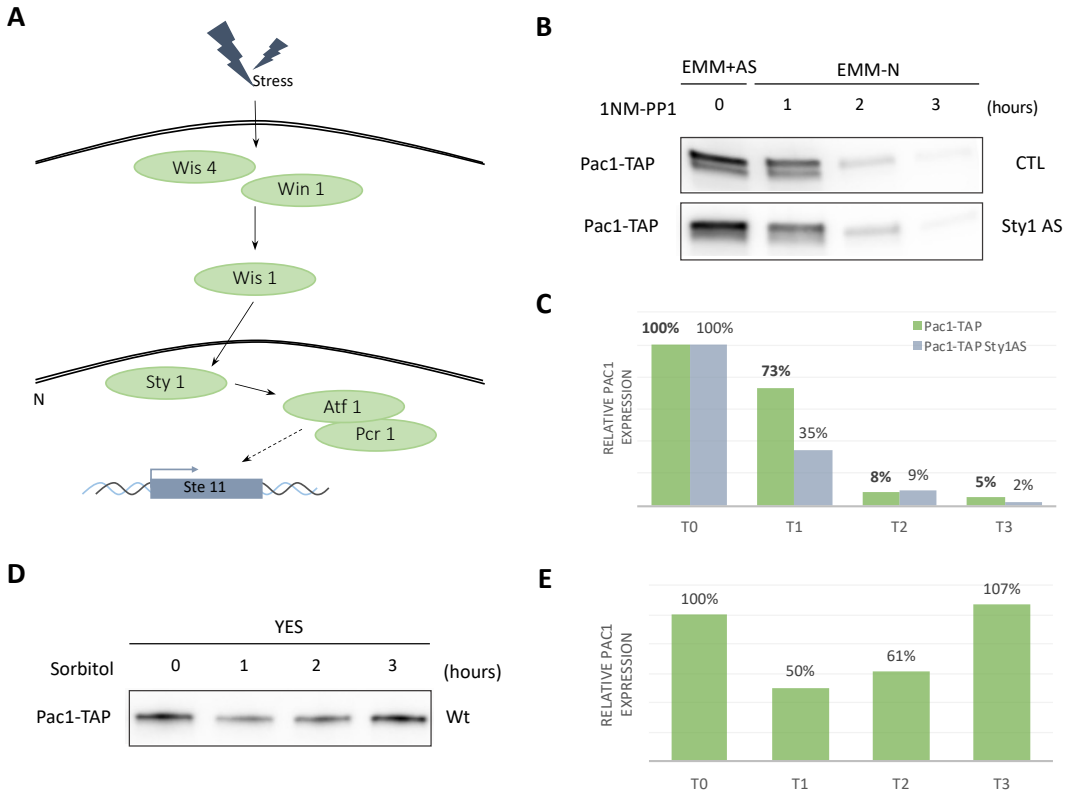

Figure 10. **The MAPK/Sty1 pathway does not regulate Pac1 abundance. A**. Pathway of the MAPK/Sty1 pathway (adapted from Otsubo & Yamamoto, 2012). Normal arrows represent an activation, dashed arrows represent indirect activation, N: nucleus. **B**. Western blot analysis of TAP-tagged Pac1 over three hours in nitrogen-free medium (EMM-N) supplemented or not with 10 µM of 1NM-PP1 for the wild-type strain (CTL) and Sty1AS strain. **C**. Quantification of the western blot in (B) (n = 1). **D**. Western blot analysis of TAP-tagged Pac1 over three hours in rich medium with osmotic stress induction (1M sorbitol) in the wild-type strain (Pac1-TAP). **E**. Quantification of the western blot in (D) (n = 1).

5. TOR-dependent regulation of Pac1

5.1. TORC2 does not contribute to Pac1 regulation in nitrogen starvation

The TOR pathway is directly involved in nitrogen sensing (Figure 11A) (Matsuo et al., 2007) and we wanted to test if one of the two complexes of this pathway, TORC1 and TORC2, is involved in the down-regulation of Pac1 in nitrogen starvation. We interfered with TORC2 by deleting its catalytic subunit (*tor1*) whose activity is important in the process of sexual differentiation (Weisman, 2016). As for the control strain, the western blot of *tor1*-deleted strain shows a decrease in the level of Pac1 during nitrogen starvation (Figure 11B, C). This result indicates that the downregulation of Pac1 in nitrogen starvation is independent of TORC2 activity.

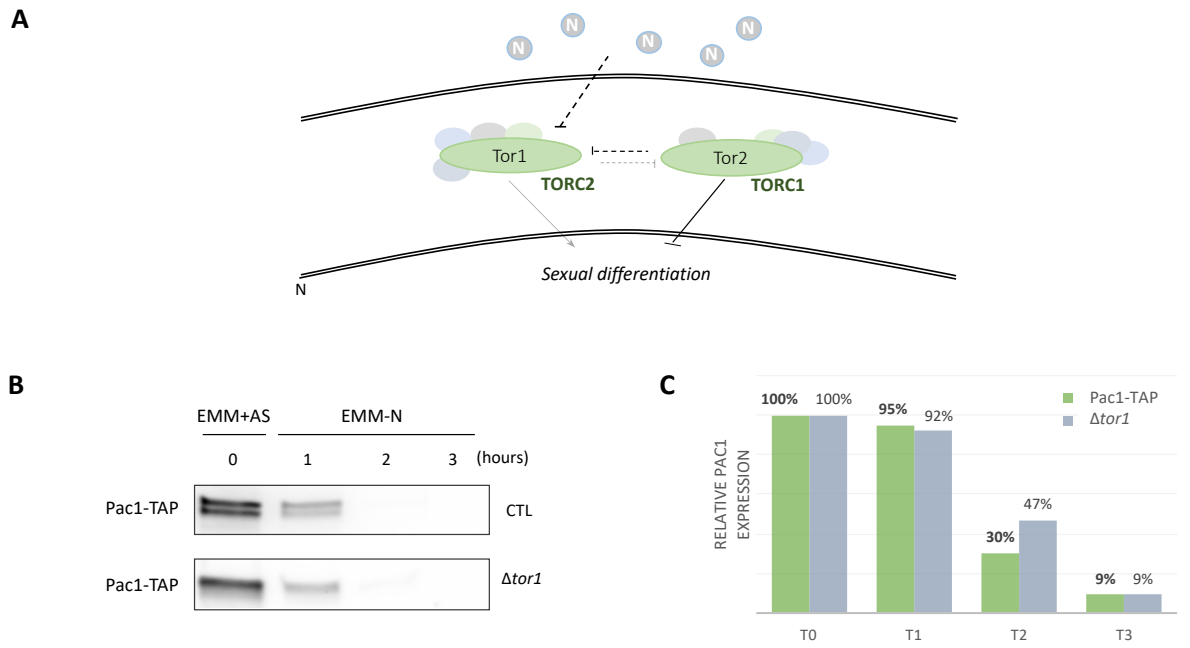

Figure 11. **TORC2 Pathway. A**. TORC2 is inhibited in the presence of nitrogen (adapted from Otsubo & Yamamoto, 2012). Truncated arrows represent inhibition, normal arrows represent activation and dashed arrows represent indirect activation, N: nucleus, circled N: nitrogen source. **B**. Western blot analysis of TAP-tagged Pac1 over three hours under nitrogen starvation condition (EMM-N) in wild-type and Pac1-TAP tor1::kanR strains. **C**. Quantification of the western blot in (B) (n = 1).

5.2. The dysregulation of TORC1 impacts the expression of Pac1

Unlike TORC2, TORC1 is inhibited by nitrogen removal (Figure 12A) (Otsubo et al., 2017). To test if TORC1 inhibition is involved in Pac1 regulation, we used a Tor2 L2048S Pac1-TAP strain, which is heat-sensitive at 37°C because contrary to *tor1*, the *tor2* gene is essential (Hayashi et al., 2007). As expected, the Tor2 L2048S Pac1-TAP strain does not grow in a nutrient-rich medium at a temperature of 37°C (Figure 12B). The western blot analysis and the quantification graph of the Tor2 L2048S Pac1-TAP strain after the temperature shift at 37°C show a decrease in the abundance of the TAP-tagged Pac1 protein (Figure 12C, D). Moreover, this decrease is seen in both rich and minimal media (Figure S5) This indicates that TORC1 inhibition is sufficient to decrease Pac1 level regardless of the medium and of the presence of nitrogen.

To further validate these initial findings, we used a Tor2 L1310P strain, in which the catalytic subunit of TORC1 remains continuously active. In this strain, Tor2 do no longer require Rhb1 to be active, so it is no longer sensitive to the negative regulation exerted during nitrogen deficiency by the TSC complex on Rhb1 (Urano et al., 2007). The analysis of the western blot and the quantification graph of the Tor2 L1310P strain in a minimal medium with proline (EMMP) shows a decrease in Pac1 abundance over the course of 3 hours similar to the control (Figure 12E, F). However, the analysis of the results shows an accumulation of pac1 at time 0, corresponding to the minimal nitrogen-rich environment. Based on these results, we can conclude that TORC1 inhibition is not required, although it is sufficient for the down-regulation of Pac1. Nevertheless, they suggest that dysregulation of TORC1 has an impact on the abundance of Pac1.

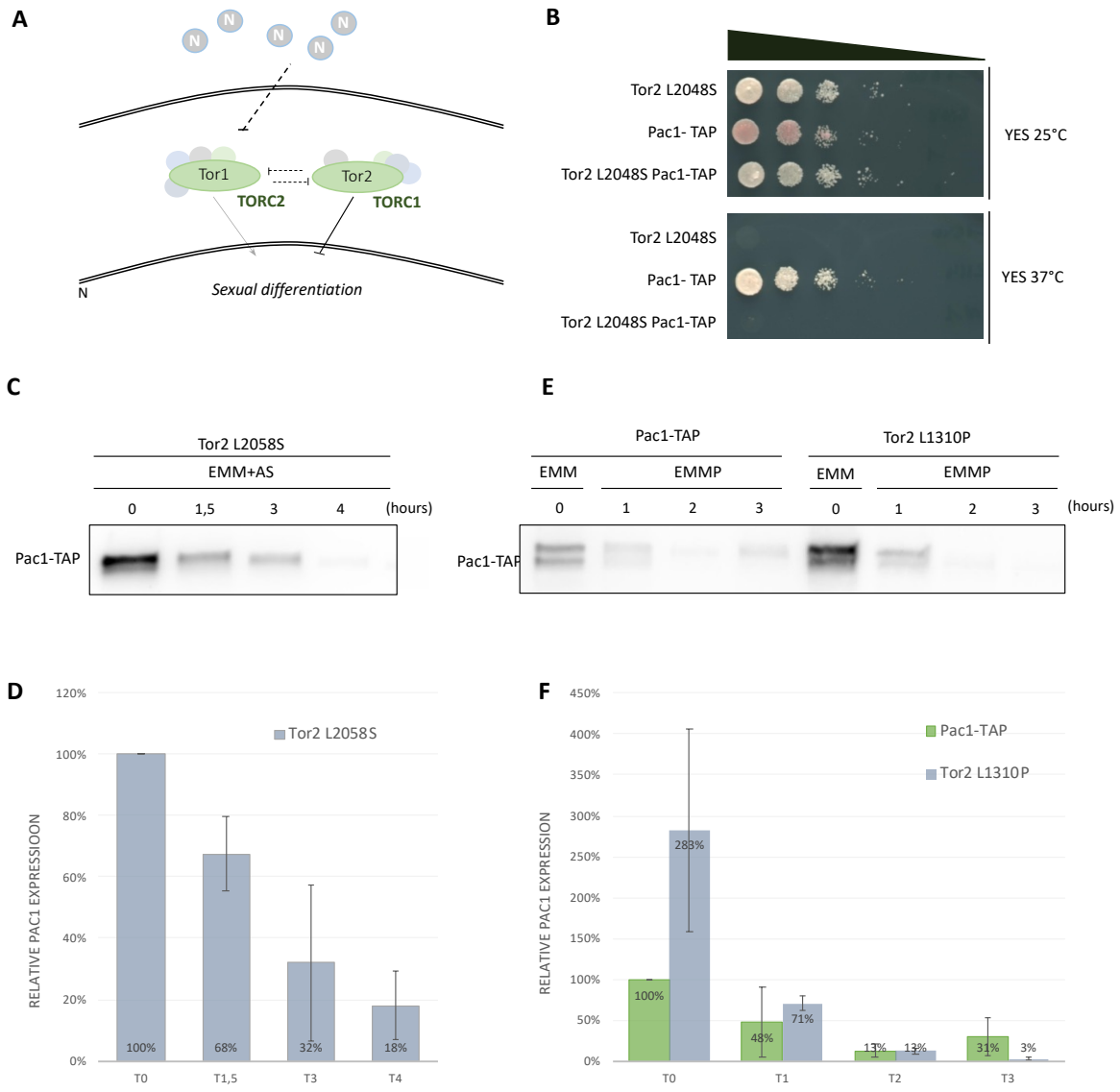

Figure 12. **TORC1 Pathway. A.** TORC2 is inhibited in the presence of nitrogen (adapted from Otsubo & Yamamoto, 2012). Truncated arrows represent inhibition, normal arrows represent activation and dashed arrows represent indirect activation, N: nucleus, circled N: nitrogen source. **B**. Drop assay of Tor2 L2048S, Pac1-TAP and Tor2 L2048S Pac1-TAP strains incubated in YES at 25°C or 37°C for three days. **C**. Western blot analysis of TAP-tagged Pac1 in the Tor2 L2048S Pac1-TAP strain, in EMM+AS over a period of 4 hours after a temperature shift at 37°C. **D**. Quantification of the western blot in (C) Error bars correspond to standard deviations (n = 2). **E**. Western blot analysis of TAP-tagged Pac1 over a period of 3 hours, after cells had shifted from EMMG (glutamic acid) to EMMP (proline) in a wild-type and Pac1-TAP Tor2 L1310P strains. **F**. Quantification of the western blot in (E) Error bars correspond to standard deviations (n = 2).

5.2.1. Activation of TORC1

As discussed in the introduction, the TORC1 complex responds to at least three different pathways. To further clarify the contribution of TORC1 into Pac1 regulation, we interfered with the TSC/Rhb1 and Gcn2-mediated pathways (Figure 13A). These pathways are positioned upstream of TORC1 and induce its repression when the cell senses a lack of nitrogen or amino acid in its extracellular environment. (Fukuda et al., 2021; Yuan et al., 2017). To confirm the

involvement of TORC1 in the regulation of Pac1, we use Δ*tsc1,* Δ*gcn2* strains and the Δ*tsc1* Δ*gcn2* mutant (Fukuda et al., 2021).

Western blot analysis and quantification graph show that the decrease in Pac1 could not be drastically stopped by deleting *tsc1* or/and *gcn2*. However, we notice a trend for the two assays carried out. After one hour of nitrogen stress, it appears that the decrease in Pac1 is slowed in the Δ*tsc1* strain. However, after two hours and three hours of nitrogen stress, we observed a decrease in Pac1 similar to the control, marked by a large variation (Figure 13B, C).

In a wild-type strain, the GTP-Rhb1 form is active and inhibited by the GAP activity of the TSC complex during nitrogen starvation (Figure 13A). Here we took advantage of a previously described mutation in Rhb1 called DA4 (Murai et al., 2009) to investigate a putative role for Rhb1 in regulating Pac1 expression. The Rhb1-DA4 activation mutant is mutated at residue 17 (V17A) within the GTP binding site. This modification affects the hydrolysis of GTP by TSC. In this way, Rhb1-GTP form induces continuous activation of TORC1 and repression of sexual differentiation. Accordingly, in nitrogen-free media, the Rhb1-DA4 strain does not respond normally to the nitrogen stress (Murai et al., 2009).

Using this mutant, we had the time to conduct two replicated experiments in which we monitored Pac1 activity during nitrogen stress. Unfortunately, the two assays conducted in minimal medium with a poor nitrogen source (EMMP) showed non-reproducible and different results: we observe either a progressive decrease of Pac1 expression, or an increase in abundance over time (Figure 13D, E and F). These contradictory results do not allow us to conclusively determine on the potential regulation of the rhb1/TORC1 pathway on Pac1 in the absence of a preferred nitrogen source. More replicates are in preparation to clarify this point. However, we still note that in both replicates, western blot analyzes show that the abundance of Pac1 is higher compared to the control when cells are cultured in a nitrogen-rich medium (time 0).

Despite the reproducibility issues (that might be attributed to aspecific protein degradation when cells activate autophagy), the involvement of the TSC/Rhb1/Tor2 pathway in the regulation of Pac1 remains an attractive hypothesis considering the accumulation of Pac1 during liquid culture in EMM in the Rhb1-DA4 (Figure 13D, E, F) and Tor2 L1310P (Figure 12E, F) mutants and the trend observed in western blot analyzes and graphic quantification carried out in EMMP in the Δ*tsc1* mutated strain (Figure 13B, C).

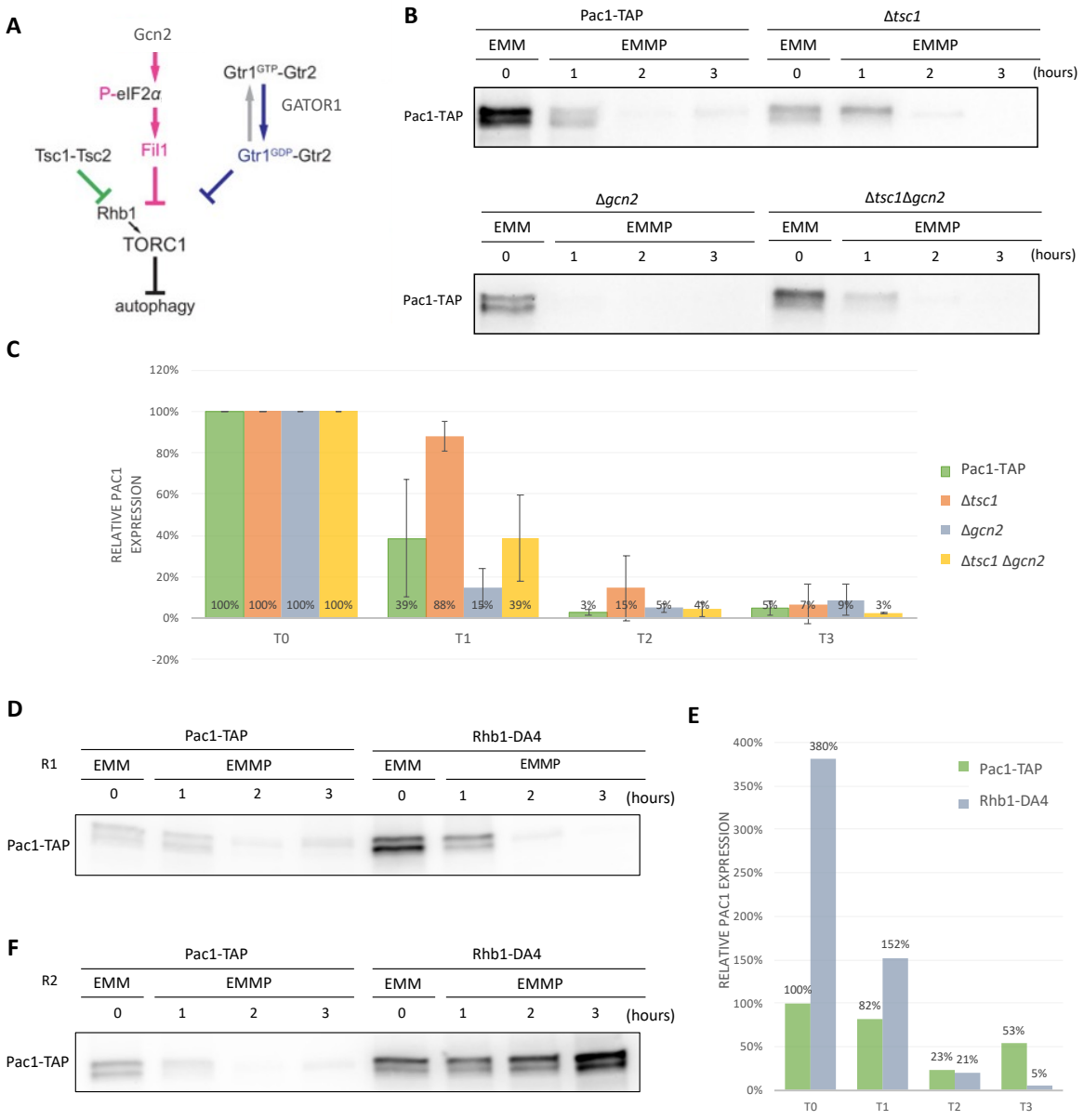

Figure 13. **TORC1 regulation. A.** Representation of TORC1 regulation orchestrated by TSC complex, Gcn2 kinase and GATOR1. Truncated arrows represent inhibition, normal arrows represent activation. **B**. Western blot analysis of TAP-tagged Pac1 over a period of 3 hours, after cells had shifted from EMMG (glutamic acid) to EMMP (proline) in a wild-type and Pac1-TAP Δ*tsc1,* Pac1-TAP Δ*gcn2* and Pac1-TAP Δ*tsc1* Δ*gcn2* strains. **C**. Quantification of the western blot in (B) Error bars correspond to standard deviations (n = 2). **D**. First assay of western blot of TAP-tagged Pac1 over a period of 3 hours, after cells had shifted from EMMG (glutamic acid) to EMMP (proline) in a wild-type and Pac1-TAP Rhb1-4DA strains. **E**. Quantification of the western blot in (D) (n=1). **F**. Second assay of western blot of TAP-tagged Pac1 over a period of 3 hours, after cells had shifted from EMMG (glutamic acid) to EMMP (proline) in a wild-type and Pac1-TAP Rhb1-DA4 strains.

6. Pac1 degradation mediated by the proteasome

Until now, our focus has been on the use of mutated strains to deregulate pathways involved in sexual differentiation and interfere with the down-regulation of Pac1. In this assay, we considered an alternative approach to interfere with the regulation of Pac1. To achieve this, we hypothesized that the regulation of pac1 during nitrogen starvation, is dependent on the proteasome (Figure 14A). This degradation would follow Pac1 ubiquitination. To test our hypothesis, we employed a proteasome inhibitor: the bortezomib. Bortezomib is notably used in antitumor treatments due to its ability to inhibit a ß subunit of the proteasome (Takeda et al., 2011; Wei et al., 2021).

Using the temperature sensitive Tor2 strain, we induced Pac1 down-regulation by inactivating TORC1 at 37°C. Western blotting reveals that the addition of various concentrations of bortezomib to the medium allows the stabilization of the heavier form of Pac1 (upper band), while the appearance of the lighter form (lower band) remains almost undetectable even with the highest concentration of Bortezomib (Figure 14B). As a control, we also assessed the impact of Bortezomib at the permissive temperature of 26°C which preserves Tor2 activity. Western blot analysis at this temperature reveals that treatment with 30 micromolar bortezomib allows the accumulation of the lighter form, with no drastic increase in the accumulation of the heavier form – a result that contrasts with what was observed at 37°C. Although this accumulation appears to be inhibitor concentration-depend, the treatment with 100 micromolar does not results in a significantly higher accumulation compared to the 30 micromolar treatment (Figure 14B, C). Based on these results (Figure 14B, C), we can conclude that Pac1 regulation by the TORC1 pathway involves degradation by the proteasome with different specificity depending on status of TORC1: when TORC1 is active, only the lighter form of Pac1 is stabilized by proteasome inhibition. Conversely, when TORC1 is inactive, it is mostly the heavier form of Pac1 that is stabilized by proteasome inhibition.

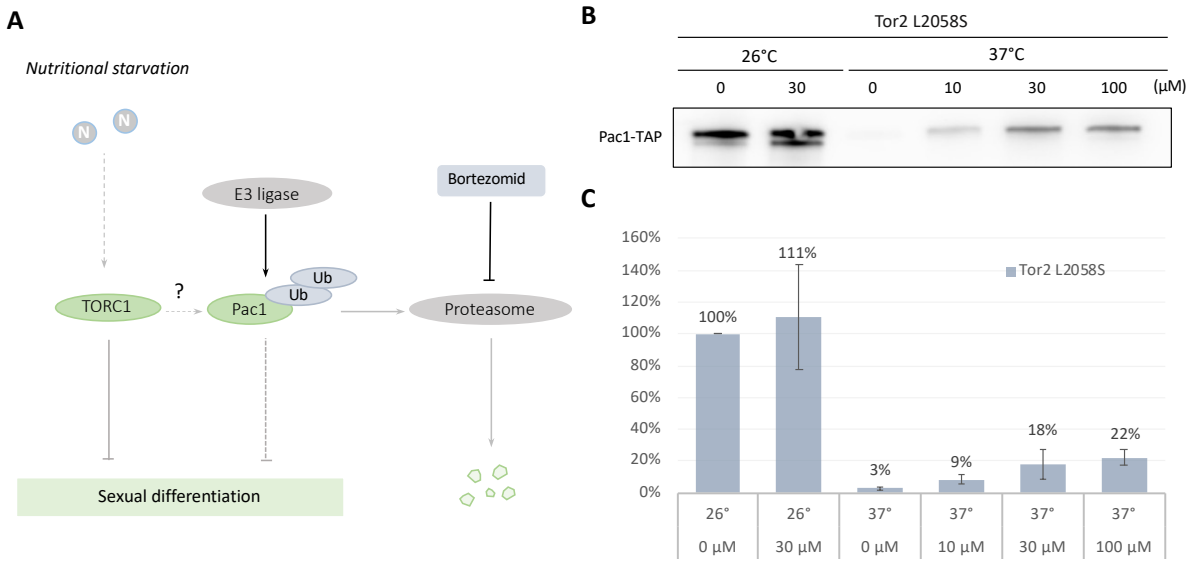

Figure 14. **Proteasome-dependent degradation of Pac1**. **A**. Working model on how the abundance of nitrogen in the environment could lead to Pac1 ubiquitination and degradation by the proteasome. **B**. Representative western blot analysis of the level of TAP-tagged Pac1 after treatment with bortezomib at a concentration of 0, 10, 30 or 100mM at 26°C or 37°C in a TORC1 thermosensitive strain. **C**. Quantification of the western blots in (B). Error bars correspond to standard deviations (n = 3 biological replicates).

7. Investigation on the nature of the double Pac1 bands

Throughout the results presented so far, we have consistently observed two specific bands of Pac1 on Western blots. Interestingly, these two bands do not always behave in the same manner. For instance, the doublet observed in the control condition decreases homogeneously over time while the western blot analysis of the Δ*tor1* strain shows a lower band more diffuse and less marked (Figure 11B). In parallel, the double band observed on the western blot performed with the Tor2 L2048S Pac1-TAP strain does not show any difference between the rich and minimal medium (Figure S5), unlike the observations previously made in Figures 9B, D and 10B, D.

The culture medium – minimal or rich medium – impacts the metabolism of the organism and its proteome (Navarrete-Perea et al., 2021). Given the differences in the double bands for Pac1-TAP that we usually observed in minimal medium (EMM), but not in rich medium (YES). Our hypothesis is based on the possible role of proteases and phosphatases (whose activity could vary depending on the culture medium) to elucidate the disparity in abundance between the bands. To do that, we treated wild-type protein extracts with phosphatase inhibitor, protease inhibitor, or both. In EMM, we observe a doublet with a more abundant slow-migrating form, independently of the treatment used. In YES, in comparison to the control, we do not observe differences between all the conditions (Figure 15A).

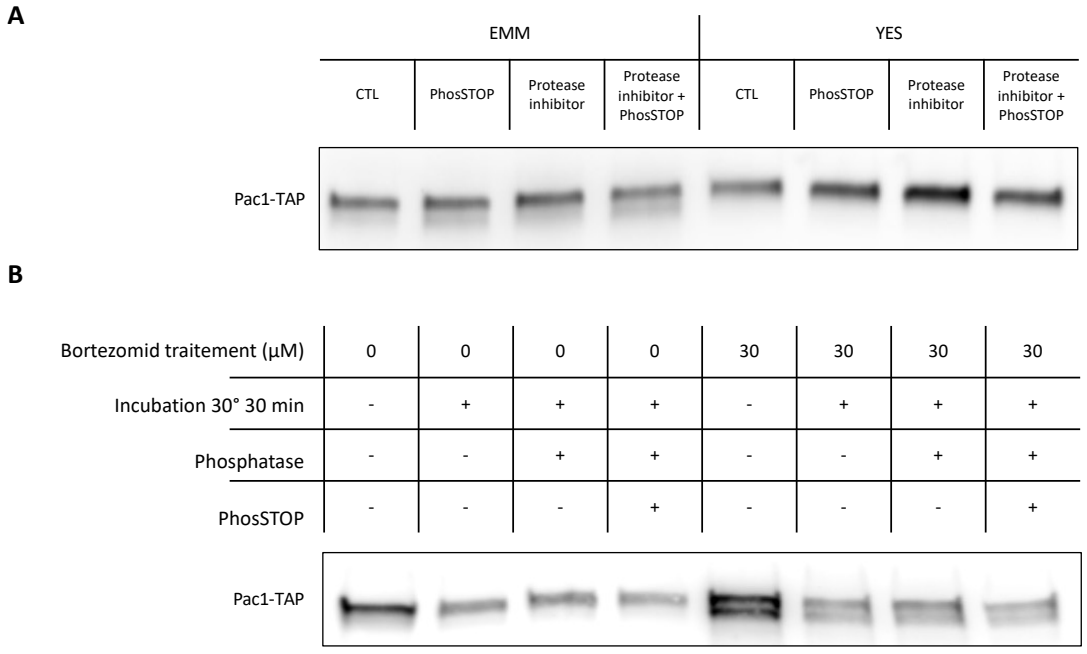

Figure 15. **Investigation on the nature of the double Pac1 bands. A**. Western blot analysis on level of TAP-tagged Pac1 in a wild-type strain cultured in minimal (EMM) or rich (YES) medium and treated during protein extraction with phosphatase and/or protease inhibitors as described in Materials and Methods. **B**. Western blot analysis on the level of TAP-tagged Pac1 in Pac1-TAP strain treated with 0 or 30µM of bortezomib. Proteins were incubated at 30°C for 30 minutes, with phosphates or/ and phosphatase inhibitors as described in Materials and Methods.

Since treatment with 30 micromolar bortezomib allows the accumulation of both forms of Pac1 (Figure 14B). A post-translational modification such as phosphorylation could direct our protein of interest toward the pathway for degradation by the proteasome or conversely allow its stability. To test this hypothesis, we treated the protein extracts with lambda phosphatase. Given that the phosphatase treatment involves incubation at 30°C for 30 minutes, we introduced a condition to verify that the heat had no impact on the resolution of our results. The use of phosphatase inhibitor serves as a second control. The resulting western blot shows a decrease in Pac1 abundance independent of phosphatase treatment (Figure 15B). Together, these results suggest that the presence of these double bands is not the result of Pac1 phosphorylation.

8. Two potentially phosphorylatable residues and protein stability

8.1. Design of pREP41-Pac1-Flag

Our main hypothesis on Pac1 regulation is grounded in the idea that a post-translational modification such as phosphorylation could enable the stability and accumulation of Pac1 in an environment favorable to vegetative proliferation. In such condition, sexual differentiation is suppressed at various levels and by the accumulation of Pac1, which acts as an inhibitor of this process. Intriguingly, the Pac1 sequence contains two sites, namely threonine 121 and serine 122, which were identified as phosphorylatable in recent proteomic studies (Halova et al., 2021; Mak et al., 2021).

In order to test the potential link between the phosphorylation and regulation of Pac1, residues potentially phosphorylatable were mutated in the Pac1 sequence. The mutated sequences were inserted into replicative plasmid (pREP) downstream of a thiamine-repressible promoter. Three versions of the plasmid exist: pREP1, pREP41 and pREP81, in decreasing order of promoter strength (Forsburg, 1993). Alanine substitutions on the putative phosphorylation sites (T121A and S122A) makes the sites unphosphorylatable. We also used phosphomimetics to mimic constitutive phosphorylation (T121E and S122E). This construction allows us to test whether these sites are involved in the regulation of Pac1. The plasmids containing mutated or wild-type Pac1-Flag insert were transfected into the main wild-type strain of our study, which contains the endogenous (chromosomal) *pac1* fused with a TAP-tag. The observation of the decrease in the abundance of Pac1-TAP during liquid culture in minimal medium with a low nitrogen source (EMMP) is used as an internal control for the nitrogen stress.

The Pac1 sequences integrated into pREP41 plasmids were sequenced and aligned with Clustal Omega to verify the presence of mutations at residues 121 and 122 (Figure 16A). The residues are positioned at the periphery of the predicted structure of Pac1, and the Flag tag is located at the C-terminus (Figure 16B). RT-qPCR analysis of the expression of Pac1 allows to confirm that the strains transformed with the plasmids are able to induce Pac1 in a thiamine-dependent manner. However, thiamine seems to have an effect on the wild-type strain, allowing better growth but also a reduction in *pac1* transcription (Figure 16C, D). The mutated strains, pREP41-Pac1-Flag, pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E S122E-Flag, also experience better growth in the presence of thiamine when the promoter is repressed. However, the plasmid seems to impact the growth of each strain (Figure 16D).

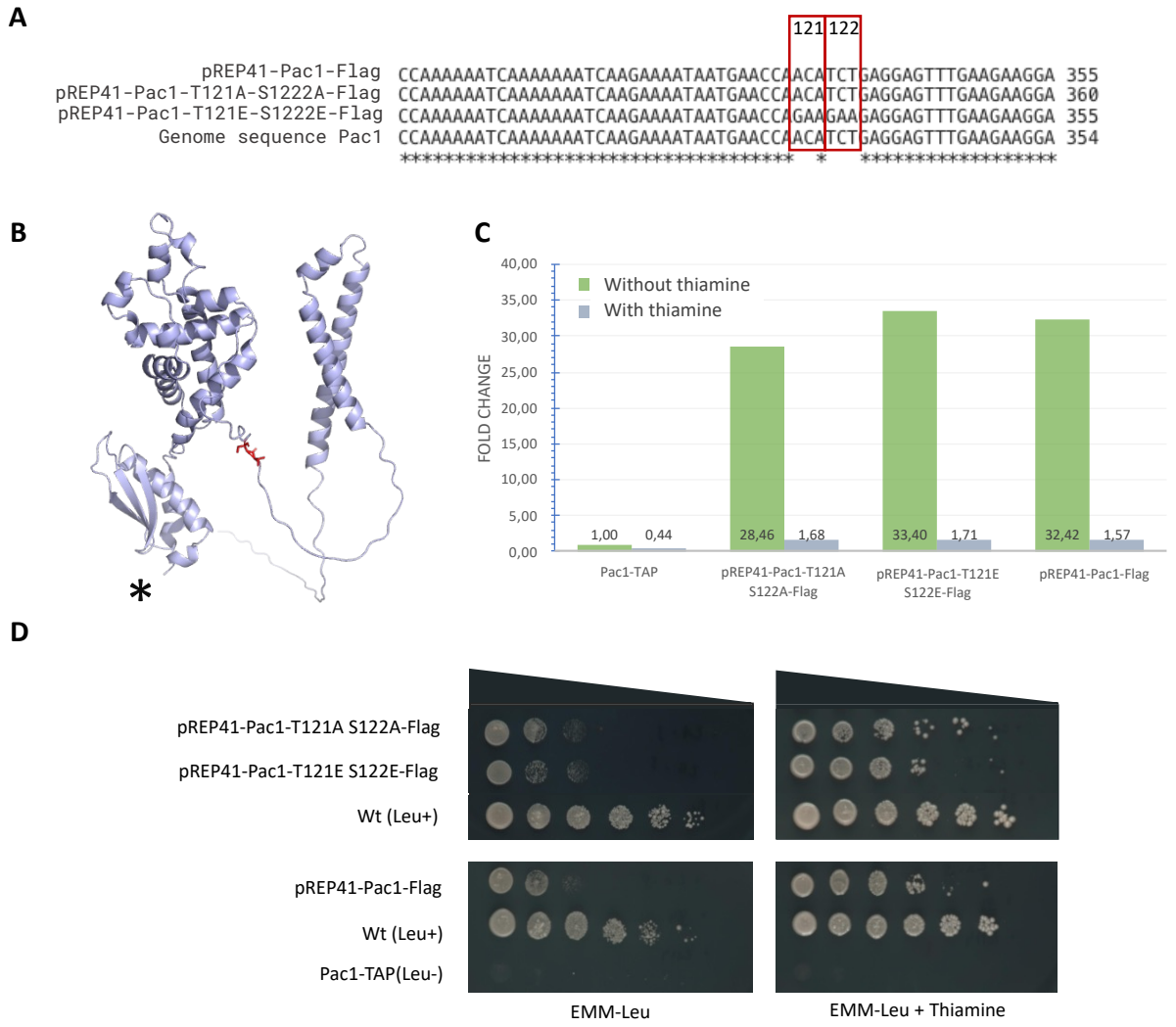

Figure 16. **Design of pREP41 Pac1-Flag strains. A.** Sequencing of Pac1 sequence. **B**. Representation of the structure of Pac1 with the mutated threonine and serine highlighted in red. (*): tag position. (PyMol) **C**. Relative *pac1* expression, in pREP41-Pac1-Flag, pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E S122E-Flag and Pac1-TAP strains incubated in EMM-leu with or without thiamine at 32°C. **D**. Drop assay of pREP41-Pac1-Flag, pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E S122E-Flag, wild-type (Leu+) and Pac1-TAP (Leu-) strains incubated in EMM-leu with or without thiamine at 32°C for three days. The strains were rearranged on Petri dishes using a photomontage to facilitate visualization (Figure S11). EMM-leu: Edinburgh minimal media without leucine.

8.2. Investigation on the impact of mutated residues on Pac1

The abundance of Pac1-TAP in the western blot resulting from the shift in EMM-leu to EMMP confirms the correct execution of the experiment. Western blot analysis shows that the abundance of Pac1-Flag does not exhibit a significant decrease over time under nitrogen starvation, for each of the mutated strains, but also for the unmutated Pac1-flag (Figure 17A). However, the two assays conducted in transfected strains did not show the same decrease in Pac1 abundance at time 3 hours (Figure 17A, B). Additionally, a second, less intense, band is observed for the plasmid-borne Pac1-flag (Figure 17A, B). This second form is similar, yet more resolved than what was usually observed for Pac1-TAP; this phenomenon can be explained by the lower molecular weight of Pac1-Flag compared to Pac1-TAP and the use of

polyacrylamide gradient gel (4-15 acrylamide %) that can provide higher resolution for smaller proteins.

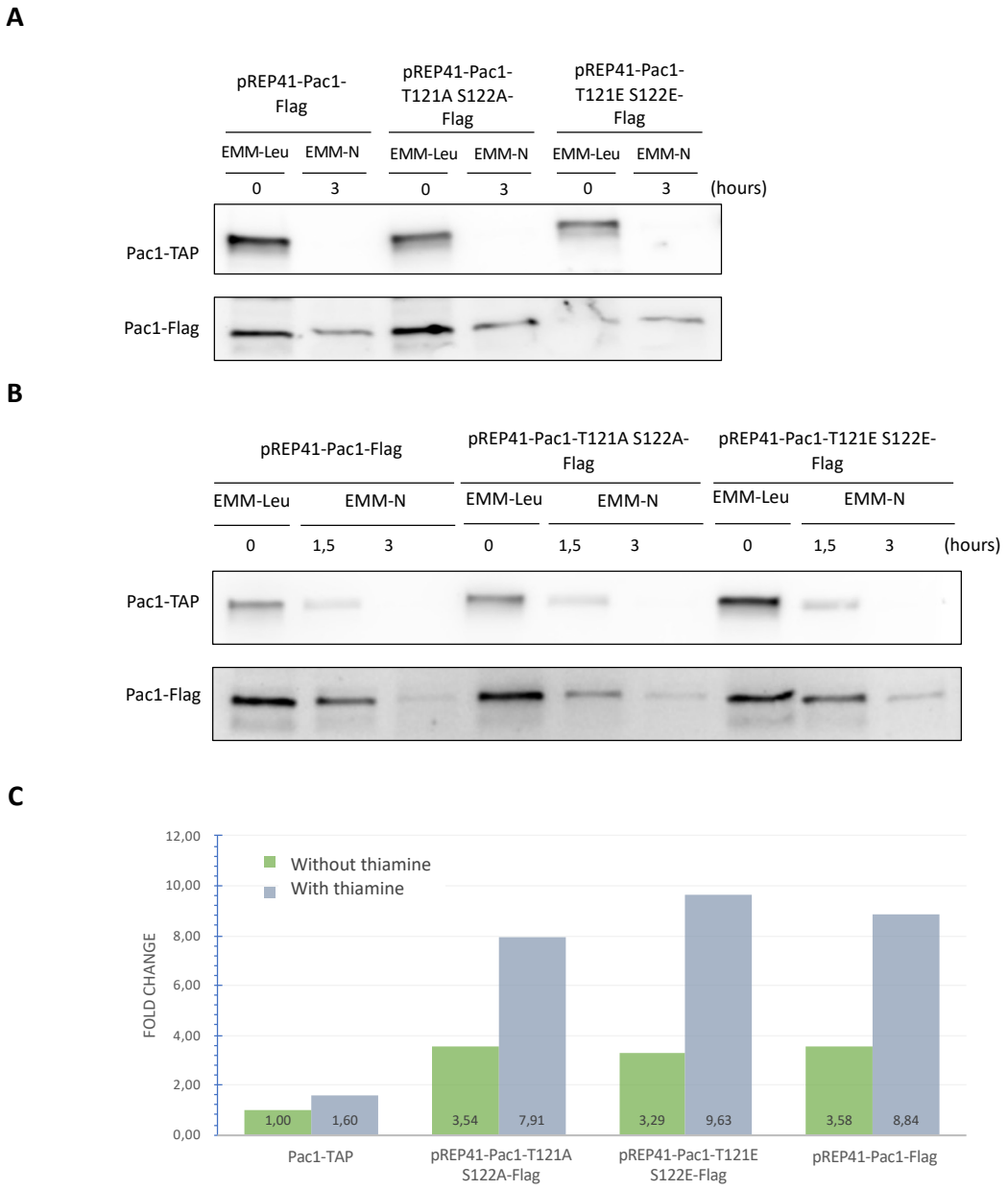

Figure 17. **Investigation on the impact of mutated residues on Pac1. A**. R1 Western blot analysis of TAP-tagged Pac1 and Flag-tagged Pac1 over a period of 3 hours under nitrogen starvation (EMM-N) in a pREP41-Pac1-Flag, pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E S122E-Flag strains. **B**. R2 Western blot analysis of TAP-tagged Pac1 and Flag-tagged Pac1 over a period of 3 hours under nitrogen starvation (EMM-N) in a pREP41-Pac1-Flag, pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E S122E-Flag strains. **C**. Relative *mfs2* expression, in pREP41-Pac1-Flag, pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E S122E-Flag and Pac1-TAP strains incubated in EMM-leu with or without thiamine at 32°C.

Figure 17C addresses a second aspect. Does constitutive phosphorylation or the absence of phosphorylation impact Pac1 in terms of its activity? To do this, we used the expression of *mfs2* as a readout of Pac1 activity. As a reminder, Pac1 is a ribonuclease that cleaves specific RNAs by recognizing secondary RNA structures in the form of stem-loop, a process that leads to the

degradation of these targets by exonucleases. *mfs2* is one of these target whose expression is therefore extremely sensitive to Pac1 activity (Yague-Sanz et al., 2021). The three transfected strains present a consistent profile in the quantification of *msf2* mRNA expression: an increase in the quantity of *msf2* expression when thiamine represses the expression of Pac1-Flag compared to conditions where Pac1-Flag is expressed and an accumulation of *msf2* RNA compared to the control strain in the presence and absence of thiamine. In parallel, the strains pREP41-Pac1-Flag, pREP41-Pac1-T121A S122A -Flag and pREP41-Pac1-T121E S122E-Flag do not present notable differences.

Firstly, these results suggest that in the transfected strains, the exogenous expression of Pac1-Flag has a deleterious effect on the activity of the protein. Secondly, when Pac1-Flag is less expressed, the degradation of *msf2* RNA is reduced. However, the interpretation of this assay is limited by the fact that we measure the activity of endogenous and exogenous Pac1, in a combined manner. Therefore, we cannot exclude that endogenous Pac1 is also repressed upon addition of thiamine or during dimer formation between endogenous and exogenous Pac1.

8.3. Design of pREP81-Pac1-HA

The unmutated Pac1-Flag does not exhibit the same response as the endogenous Pac1-TAP to nitrogen starvation. Moreover, we did not obtain reproductive results (Figure 17A, B). We considered that the Flag-Tag could interfere with the protein as transfection with the plasmid carrying Pac1-Flag is deleterious for growth (figure 16D) and Pac1-Flag has compromised activity (figure 17). Therefore, we chose to utilize an HA-tag with a flexible three-glycine linker (Figure 18B).

To decrease the transcription level of our exogenous protein so that it matches more closely the endogenous level of expression of *pac1* (and limit potential dominant negative effect), we transformed the Pac1-TAP strain with the Pac1 HA-tagged sequence under the control of a weaker version of the thiamine-repressible promoter: the pREP81 plasmid. Additionally, the Pac1 sequence has been mutated at threonine 121 et serine 122 as detailed in Chapter 8.1. Furthermore, we transfected the pREP81 plasmid lacking the Pac1 HA-tagged sequence. This strain will serve as a control for assessing the growth and impact on the plasmid on *S. pombe*.

The sequences of Pac1 integrated into the plasmids were sequenced again and aligned with Clustal Omega to verify the presence of the desired mutations on residues 121 and 122 and to ensure that the sequences did not exhibit other mutations (Figure 18A). Using a primer specific to the nucleotide sequence encoding the HA tag, the RNA quantification of Pac1-HA (Figure 18C) shows that the promoters undergo thiamine-induced repression as desired. However, RNA Pac1-HA appears less abundant in the pREP81-Pac1-HA strain compared to the pREP81-Pac1-T121A S122A-HA and pREP81-Pac1-T121E S122E-HA strains. Furthermore, as expected, Pac1-HA RNA is not detected in our control strain, which was transformed with a plasmid lacking the sequence of our protein of interest. The transfected strains exhibit a growth defect (Figure 18D). Indeed, the control strain transferred with a plasmid lacking the sequence seems to be the most impacted by this plasmid insertion. However, all strains combined reach a plateau at an OD_{595} of between 0.2 and 0.4.

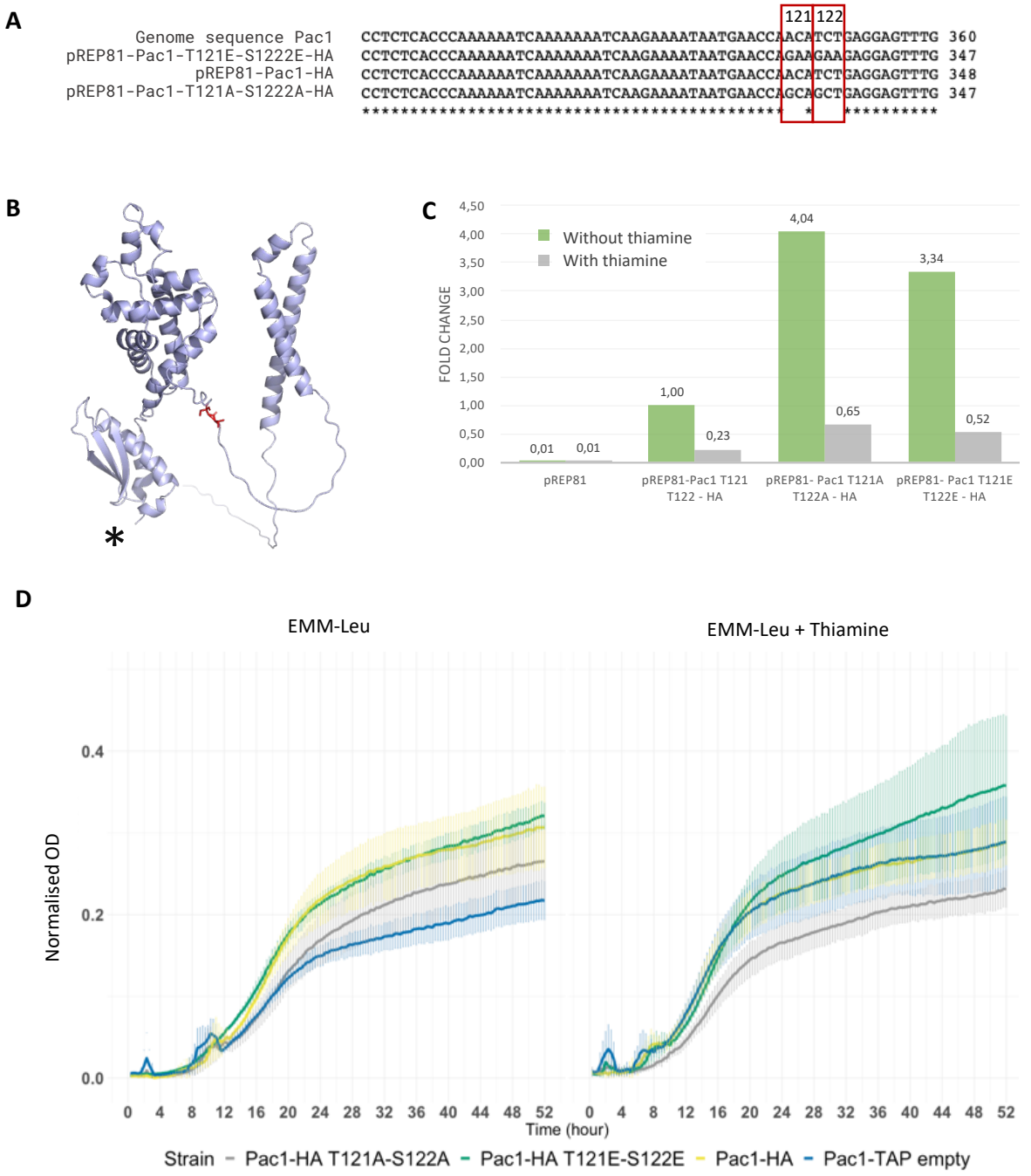

Figure 18. **Design of pREP81 Pac1-HA strains. A.** Sequencing of Pac1 sequence. **B**. Representation of the structure of Pac1 with the mutated threonine and serine highlighted in red. (*): tag position. (PyMol) **C**. Relative *pac1-HA* expression, in pREP81-Pac1-HA, pREP81-Pac1-T121A S122A-HA, pREP81-Pac1-T121E S122E-HA strains and Pac1-TAP strain transformed with an empty plasmid incubated in EMM-leu with or without thiamine at 32°C. **D**. Growth curves of pREP81-Pac1-HA, pREP81-Pac1-T121A S122A-HA, pREP81-Pac1-T121E S122E-HA strains and Pac1-TAP strain transformed with an empty plasmid incubated in EMM-leu with or without thiamine at 32°C for three days. EMM-leu: Edinburgh minimal media without leucine.

8.4. Investigation on the impact of mutated residues on Pac1

The western blots performed during nitrogen stress (EMMP) reveal a gradual decrease over time in Pac1-TAP, as previously observed in the different western blots carried out during this research (Figure 19A, Figure 7). Regarding the exogenous Pac1-HA protein, Western blot analysis shows a decrease over time, however this decrease is not complete (Figure 19A). The presence of Pac1-HA after 3 hours in the strains transfected with pREP81-Pac1-T121A S122A-HA and pREP81-Pac1-T121E S122E-HA can be explained by the basal expression level of the protein. Notably, there is a lower abundance of Pac1-HA for the strain transfected with the pREP81 plasmid containing the non-mutated Pac1 sequence compared to the other transfected strains. This difference can be related to the lower RNA level observed this strain compared to the pREP81-Pac1-T121A S122A-HA and pREP81-Pac1-T121E S122E-HA strains (Figure 18C).

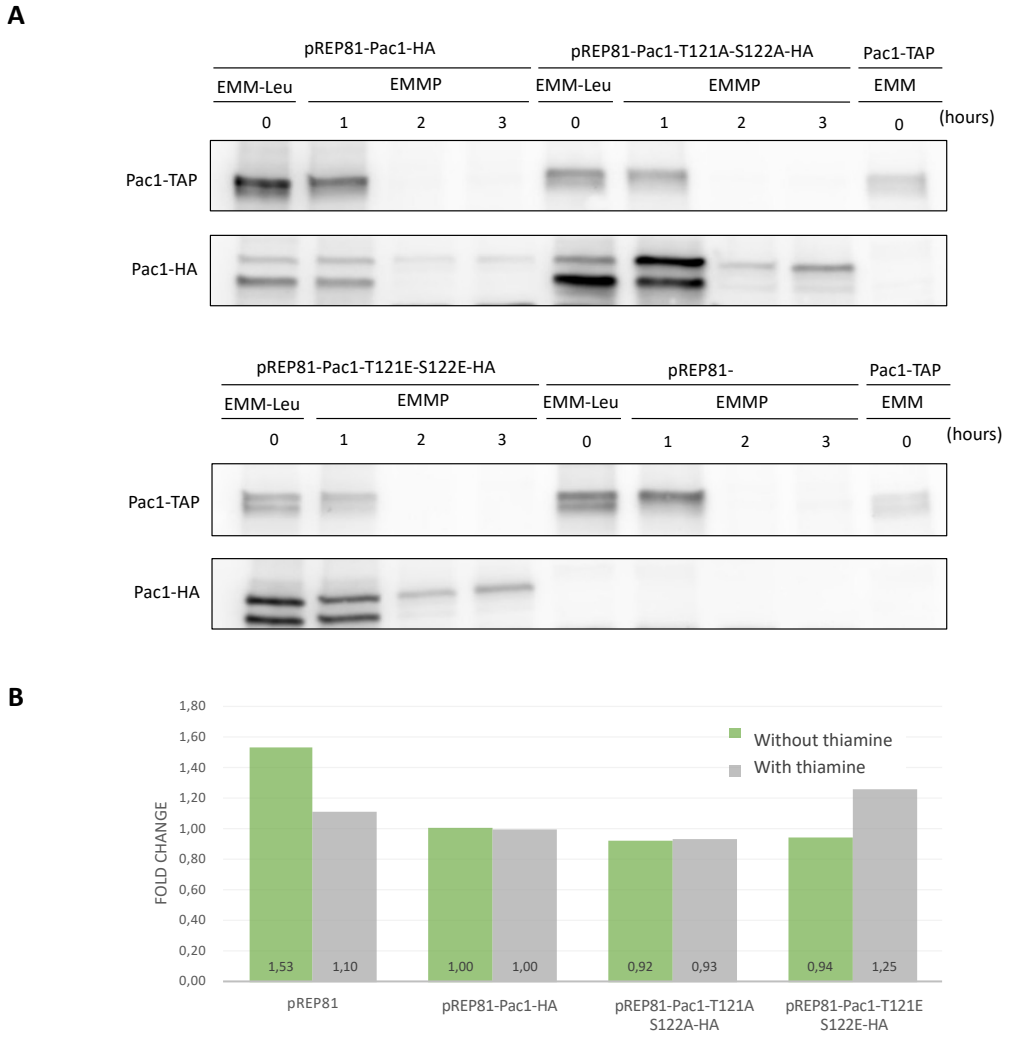

Figure 19. **Investigation on the impact of mutated residues on Pac1. A**. Western blot analysis of TAP-tagged Pac1 and HA-tagged Pac1 over a period of 3 hours, after cells had shifted from EMMG (glutamic acid) to EMMP (proline) in a pREP81-Pac1-HA, pREP81-Pac1-T121A S122A-HA, pREP81-Pac1-T121E S122E-HA strains and Pac1-TAP strain transformed with an empty plasmid. **B**. Relative *mfs2* expression, pREP81-Pac1-HA, pREP81-Pac1-T121A S122A-HA, pREP81-Pac1-T121E S122E-HA strains and Pac1-TAP strain transformed with an empty plasmid incubated in EMM-leu with or without thiamine at 32°C.

An interesting observation is the difference in abundance between the two putative forms of Pac1. The heavier form shows a slower decrease over time during nitrogen stress.

To assess Pac1 activity, we quantified *mfs2* expression, a mRNA target by Pac1 for degradation. First, in the strain transfected with an empty plasmid we observe an effect of thiamine on Pac1-TAP activity. Secondly, for the strains pREP81-Pac1-HA, pREP81-Pac1-T121E S122E-HA and pREP81-Pac1-T121A S122A-HA, the quantity of *msf2* RNA remains stable regardless of the inhibition of the promoter by thiamine (Figure 19B). This indicates that Pac1 total activity is not compromised by the expression of exogeneous Pac1-HA, contrary to what happens with the overexpression of Pac1-flag (Figure 17C). However, even with this better expression system, we still have some issues (growth delay and basal RNA expression differences between the control and mutated *pac1-HA*) and are unable to conclude on a role for the putative phosphorylation sites T121 and S122.

Discussion

In this project, we investigated the regulation of Pac1 under nitrogen starvation. However, this study is part of a broader perspective, being an integral part of a research project aimed at describing and studying the role of Pac1 during sexual differentiation in *Schizosaccharomyces pombe*. Pac1 is recognized as an inhibitor of this process and its regulation, which occurs when the cell perceives a lack of nitrogen in its extracellular environment, a condition conducive to sexual differentiation. However, the precise role of Pac1 and the mechanisms governing ribonuclease remain largely unknown.

1. Regulation of Pac1

With the aim of studying the down-regulation of Pac1, we produced different mutants to mimic nitrogen deficiency or block the induction of pathways involved in sexual differentiation. Among these pathways, we chose not to analyze the pheromone-sensing pathway that is classically induced in sexual differentiation. The reason for this is that our original observation that Pac1 is down-regulated in nitrogen starvation (Figure 7) was made using a heterothallic strains. Heterothallic yeasts cannot find conjugation partner in the same culture because the pheromone produced is not compatible with the pheromone receptor expressed. Since Pac1 was down-regulated in nitrogen starvation in such a strain, we concluded that the regulation operated on Pac1 was independent of the pheromone sensing pathway.

We also have shown that the regulation of Pac1 in nitrogen starvation is operated independently of the transcription factor Ste11 (Figure 8), Ste11 being a central player mediating the activation of many actors necessary for sexual differentiation, we reasoned that the regulation operated on Pac1 could come from one of the signaling pathways upstream of Ste11 (excluding the pheromone sensing pathway): the TOR pathway, cAMP/PKA pathway or the stress activated MAPK pathway. Our results indicate that the MAPK pathway mediated by Sty1 and cAMP/PKA pathway do not contribute to the regulation of Pac1 expression (Figure 9 and 10).

1.1. Tor-dependent regulation of Pac1?

The TOR signaling pathway is a direct sensor of nitrogen stress. While the *tor1* mutant does not influence the regulation of Pac1, Tor2, the catalytic subunit of TORC1 might be involved. Indeed, preliminary data (Figure 12) show that it is possible to mimic the effect of nitrogen starvation on Pac1 expression by inactivating Tor2, even in rich medium. In addition, overactivation of Tor2 or Rhb1 promotes the accumulation of Pac1 in nitrogen-containing media (Figure 12 E, F, Figure 13D, E, F; T0). This data supports the importance of Tor2 in the regulation of Pac1. However, during nitrogen stress, the hyperactivation of Tor2 was generally not sufficient to prevent the degradation of Pac1 over time. We still noted that the Tsc1 pathway influences the accumulation of Pac1 during early nitrogen stress conditions although the impact is modest (Figure 13B, C).

Like Pac1 overexpression, Tor2 overexpression inhibits sexual differentiation. The cells still experience a G1 arrest but do not continue the process of differentiation, mating and spore formation by meiosis. Otsubo *et al*., note that in a rich-nutritional medium, a Tor2-ts mutant presents an abnormally high level of mating which is explained by the lifted inhibition exerted

by Tor2 on the process of sexual differentiation, and in particular with the hypo-phosphorylated form of Mei2. However, *mei2* deletion in *tor2-ts* does not fully restore a normal mating frequency (Otsubo et al., 2014). This suggests that one or more actors dependent on Tor2 intervene in the regulation of sexual differentiation besides the direct regulation of Mei2. In regards with our result, Pac1 would constitute a good candidate. Indeed, we hypothesize that in the *tor2-ts* mutant condition, Pac1 is degraded and is no longer able to negatively regulate sexual differentiation, which would allow the mating events.

How could Tor2 regulate Pac1 expression? A possible mechanism has been described recently in Wei *et al*. They found that Tor2 within TORC1, which is activated in a condition of nutritional abundance phosphorylates one of its target proteins, Pir1 and that this phosphorylation allows Pir1 stability. Indeed, when Pir1 is not phosphorylated, the protein is polyubiquitinated by an E3 ligase, which leads Pir1 on the way to degradation by the proteasome (Wei et al., 2021). A similar mechanism could be at play for Pac1. Supporting this hypothesis: bortezomib, a proteasome inhibitor, allowed Pac1 accumulation in a TORC1 inhibition-induced downregulation (Figure 14B, C).

In this project, it is necessary to distinguish between the regulation of Pac1 during nutritional stress, where Pac1 is degraded, and a basal condition where Pac1 appears stable. Considering this model, when the environment is sufficiently nutritious, Pac1 could be stabilized in a Tor2-dependent manner (either through direct phosphorylation or indirectly). The presence of Pac1, on basal condition, allows the cell not to enter into sexual differentiation, which would maintain the cell on vegetative division (Figure 20A).

Excluding the contradictory results observed during nitrogen stress in the Pac1-TAP Rhb1-DA4 strain (which will be discussed below), the second part of the model focuses on the regulation of Pac1 when the cell senses a lack of nitrogen (Figure 20B). By reconsidering once again the model proposed by Wei *et al*., when the cell senses a lack of nitrogen, Tor2 is inactivated and no stabilize Pac1. However, even although we identified TORC1 as a potential regulatory pathway, our results indicate that this pathway is not strictly required for the downregulation of Pac1 during nitrogen starvation, suggesting the involvement of other mechanisms. Therefore, we can hypothesize the presence of an additional factor, whose expression or activation could depend on nitrogen starvation. Indeed, in nitrogen-rich conditions, our data shows that disruption of the TOR pathway is sufficient to prevent Pac1 degradation. In response to a nitrogen deficiency, this second factor would be activated and play an inhibitory role on Pac1. Finally, Pac1 would be subject to degradation by the proteasome. The proposed model would be in line with the sterility observed during an overexpression of Pac1.

The second factor may arise from the rearrangement of gene expression during sexual differentiation. It is important to note that many genes are regulated by Ste11, a key transcription factor. Our results (Figure 8B, C) indicate that Ste11 is not essential for the down-regulation of Pac1. However, we hypothesize the potential coordination of Pac1 regulation by both Ste11 and TORC1. Indeed, interference with these factors individually does not seem sufficient to inhibit the down-regulation of Pac1 (Figure 8 and 12). Nevertheless, deleting Ste11 in a strain with active TORC1 could allow us to investigate whether this regulation is subject to the influence of both factors in parallel. An alternative hypothesis, which involves the activation of this factor through a process dependent on nitrogen starvation, is also considered.

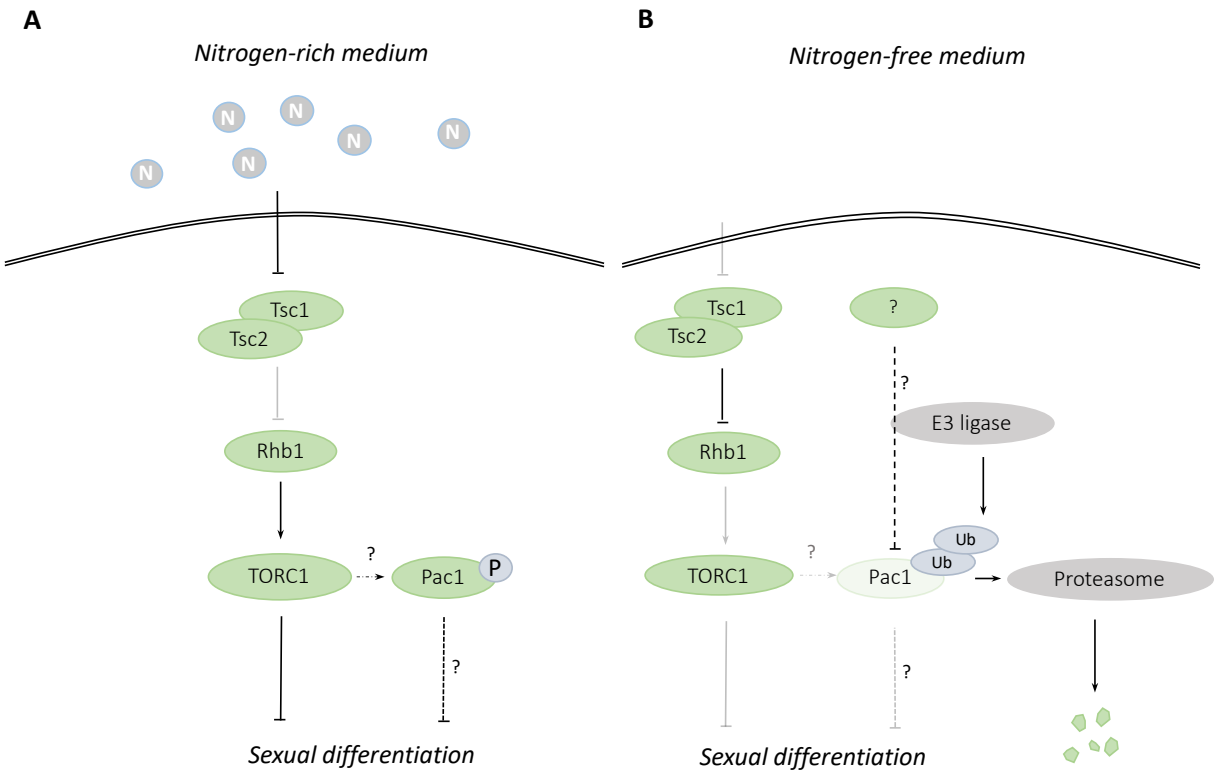

Figure 20. **Model of Pac1 regulation**. **A**. In nitrogen-rich medium the TORC1 complex allows the stabilization of Pac1. **B**. In a nitrogen-free medium, TORC1 is no longer active, and Pac1 is no longer stabilized. Pac1 is finally led down the degradation pathway. In addition, a second actor induces repression of Pac1.Yeast can enter sexual differentiation. Truncated arrows represent inhibition, normal arrows represent activation and dashed arrows represent indirect activation, circled N: nitrogen source.

Threonine 121 (T121) and serine 122 (S122) were identified as potentially subject to phosphorylation, based on a phosphoproteomic study (Halova et al., 2021; Mak et al., 2021). Upon examination of the predicted three-dimensional structure of Pac1, we note that these residues are peripherally located, facilitating access for phosphorylation (Figure 16B). We postulated that these residues could play a preponderant role in the regulation of Pac1, thus contributing to its stability. Given that Pac1 is an essential gene, we opted to interfere with this phosphorylation on exogeneous copies of Pac1. Various strains were transfected with plasmids containing mutations that made these positions either non-phosphorylatable (T121A S122A) or mimicked continuous phosphorylation (T121E S122E). However, the introduction of plasmids appears to have deleterious effects on the strains, leading to a certain variability between assays. To advance our hypothesis, we intend to induce these mutations directly in the *S. pombe* genome, using the CRISPR method. However, a significant concern persists: the essential nature of Pac1. If we prevent the stability of Pac1, it is possible to obtain no clones during the CRISPR assay. Accordingly, while this manuscript was in preparation, we successfully obtained the endogenous Pac1 T121A S122A but failed to obtain the T122E S122E mutant. Characterization of the obtained mutant is ongoing.

2. Investigation on the nature of the double bands

Regarding the presence of the double band for Pac1-TAP in western blot, the presence of the bands is not impacted by treatment with phosphatase inhibitors, protease inhibitors of

lambda phosphatase (Figure 15) and might therefore not result from phosphorylation. We noticed that the presence of the double band is linked with the growth medium: in YES medium we only see one band while in EMM we usually see two bands. However, the relative intensity of the two bands in EMM vary from experiments to experiments. To explain this discrepancy, we are considering different avenues, such as a variation of material used and the impact of the culture medium on the cell proteome, or on the metabolism (Navarrete-Perea et al., 2021). A promising explanation can be found in Thodberg *et al*., who mapped transcription start sites (TSS) across the entire *S. pombe* genome. It appears that Pac1 has two TSSs whose relative usage depends on the growth medium. In a minimal medium, there would be an alternative transcription start site located upstream of the coding part and a major site in the coding sequence of *pac1*, with a distance almost 1kb separating them. A nitrogen-free medium does not impact the TSSs compared to the minimal medium, the RNAs have the same transcriptional profile. In a nutrient-rich medium, only one site is used to initiate transcription, the distal one upstream of *pac1* (Thodberg et al., 2019). These different transcriptions start sites could give rise to the two *pac1* transcript isoforms of 1.4 and 2 Kb previously describes (Lino 1991). In turn the two RNA isoforms could be translated in protein isoforms of different size, which could explain the difference in migration observed on western blot analyses.

In addition to the observed variation depending on the environment conditions. In a minimal culture, where Pac1 transcription is initiated at two TSSs, we generally observe a difference in abundance between the two forms of Pac1. Indeed, the majority form is generally the heaviest form. Furthermore, our results show that the accumulation of the lighter form can be induced when the proteasome is inhibited by bortezomib.

In a minimal medium culture, the TSS leading to the shortest transcript is located downstream of the annotated start codon part of Pac1. It is important to highlight that the predicted dimerization site of Pac1 is located in the N-terminal region. This distinction suggests that the shorter isoform might have distinct dimerization properties, due to a truncated dimerization site. The coexistence of these two forms opens up new prospects, such as a decrease in the activity of the truncated form leading to proteasome-controlled degradation, or a differentiated role between the two forms of Pac1. Interfering with these start codons or creating strains expressing only the truncated or long form, could enable us to study and validate the presence of a short form and a long form, and ultimately the difference in stability and functionality between the two forms of Pac1.

3. Technical issue : protein degradation during nitrogen stravation

Western blots analysis has revealed that our samples are subject to degradation (Figure 7). Indeed, we observe an increase in degradation in response to nitrogen starvation. This phenomenon can be attributed to various factors. Firstly, autophagy is induced by the inactivation of TORC1 and is required during nitrogen starvation (Corral-Ramos et al., 2022). This is a catabolic process aims to reorganize the proteome. The material such as proteins are transported to the vacuoles to be fragmented and released as amino acids (Corral-Ramos et al., 2022; Weisman, 2016). The variable induction of autophagy during our assays constitutes a first factor. Secondly, during the protein extraction step, membrane lysis releases all the proteins present in different organelles, exposing them to degrading enzymes. Although protein extraction is performed on ice, we hypothesize that the activity of proteases is not completely abolished.

Concerning the increased degradation observed in a nitrogen-free medium, it is important to highlight that experiments investigating the regulatory role of the cAMP/PKA pathway and the MAPK pathway on Pac1 were notably carried out in a nutrient-rich medium (Figure 9D and 10D). Blots generated from cultures in this enriched medium consistently demonstrated an absence of degradation, further validating the obtained results and supporting the conclusions.

Throughout this Master thesis, we have attempted to manage this technical issue by utilizing various culture media. It was observed that the use of a poor nitrogen source, such as proline, resulted in less degradation of total proteins compared to a complete lack of nitrogen (Figure 7). Implementing this method mitigated the phenomenon for numerous samples. Nevertheless, we continue to observe a variation in degradation. This variation is particularly noticeable among replicates. During nitrogen stress assays in the Pac1-TAP Rhb1-DA4 strain, we observed different results. However, the analysis of the first membrane indicates that the proteins appear to have undergone degradation (Figure 13D, E, Figure S7), compared to the second membrane (Figure 13F, Figure S7). Furthermore, the decrease in Pac1 abundance is observed concurrently with protein degradation.

All our protein extractions were performed by mechanical lysis with zirconium beads as described in the 'Protein extraction' section of the Materials and Methods. Grallert and Hagan developed a protein extraction technique in *S. pombe* based on mechanical lysis (zirconium or glass beads) and the use of protease inhibitors and a precipitation agent. Firstly, phenylmethylsulfonyl fluoride (PMSF), a protease inhibitor, is added to the culture before centrifugation for 4 minutes at 4000 rpm. Secondly, trichloroacetic acid (TCA) is used prior to cell lysis. It is employed to precipitate and denature proteins, eliminating enzymatic activity and fixing the protein content (Grallert & Hagan, 2017).

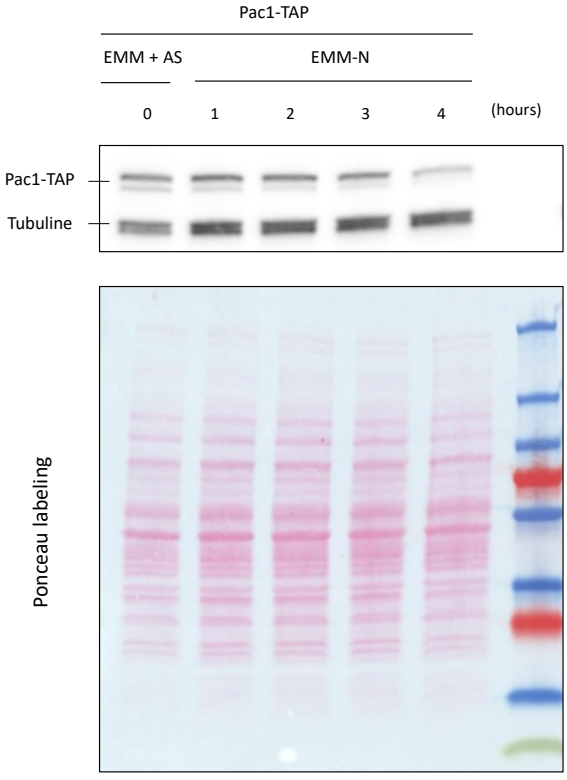

Figure 21. **Protein extraction**. Western blot analysis of TAP-tagged Pac1 over a period of 4 hours under nitrogen starvation in a wild-type strain. Proteins extracted according to Grallert and Hagan protocol. Labeling of total proteins using Ponceau labeling.

This technique confirmed the progressive degradation of Pac1 under nitrogen starvation (Figure 21). However, it is important to note that this degradation is characterized by a slower decline, with no dramatic decrease observed after 4 hours. Total proteins stained with Ponceau labeling show no degradation, suggesting that Pac1 degradation results from down-regulation induced by nitrogen starvation.

Using this new protein extraction, based on the Grallert and Hagan protocol, drastically reduces the degradation of the proteins present in our samples (Figure 21). The results previously obtained will be tested once again using this technique to verify our initial hypotheses, and the involvement of TORC1 in Pac1 downregulation.

4. Application in superior eukaryotes cells

There are several Pac1 homologs, notably in humans, such as the RNase Drosha. Drosha is widely studied for its role in microRNA maturation. MicroRNAs are small, single-stranded, non-coding RNAs. They play a post-transcriptional regulatory role, repressing the RNAs to which these miRNAs hybridize. During biogenesis, these RNAs adopt a hairpin structure, allowing them to be cleaved by Drosha. The maturation process of these cleaved RNAs (pre-miRNAs) is finalized in the cytoplasm (Han et al., 2004; Lee et al., 2006).

In this Master thesis, we investigated the regulation of TORC1 by Pac1. However, the regulation of Drosha (Pac1 homolog) induced by TOR has been investigated in human cells (Ye et al., 2015). Activation of mTOR enables the regulation of numerous genes such as *mdm2*. Mdm2 is an E3 ubiquitin ligase, whose targets include Drosha. The ubiquitinated form of Drosha is degraded by the proteasome. This degradation leads to a reduction in miRNA abundance. In contrast, inactivation of mTOR allows miRNA accumulation (Ye et al., 2015). This study is particularly relevant to cancer research. Indeed, the characterization of mTOR is particularly important in the study of cancer, due to its regulation of cell proliferation. Activation of the TOR pathway is generally described in cancer cells (Pópulo et al., 2012). This interest in cancer research is also linked to the involvement of miRNAs in various cancers. Among miRNAs, some are considered tumor suppressors and are downregulated in cancer cells (Garzon et al., 2009).

In conclusion, this indirect regulation by mTOR on Drosha leads to the degradation of the RNase. While this mechanism contrasts with the one presented in this thesis, we still observe a highlighted interaction between these actors. Therefore, using the fission yeast as a model might allow to better understand the regulation of the RNAse III homologs in other species, including mammals.

Materials and methods

Culture condition, yeast strain

Under standard conditions, the strains used in this work (Table 1) were cultured in yeast extract medium containing 3% glucose (YES), or in Edinburgh minimal medium (EMM) containing 2% glucose and supplemented with adenine, uracil, leucine and histidine (225 mg/L) (EMM + AS), depending on the strain.

Table 1: List of the strains used in this Master thesis.

Database Number	Name	Description	Genotype	Reference
94	Wild-type	Wild-type	h-	Nurse
568	Wild-type	Wild-type	h90	Nurse
1142	Sty1as	Sty 1 Analogue Sensitive	h-; sty1 T97A	(Zuin et al., 2010).
1640	Tor2 L2048S	Tor2 thermo sensitive	h-; tor2 L2048S-kanR	(Hayashi et al., 2007)
1664	tcs1 :: kanR	tsc1 deleted	h-; tsc1 ::kanR	(Laboucarié et al., 2017)
2114	Pac1-TAP	Pac1 TAP-tagged (Wild-type on this study)	h+; pac1-TAP-natR; ade6-210; ura4-D18; leu1-32; his3-D1	(Yague-Sanz et al., 2021)
2185	Pac1-TAP ste11 :: kanR	Pac1 TAP-tagged ste11 deleted	h+; pac1-TAP-natR; ste11::kanR; ade6-210; ura4-D18; leu1-32; his3-D1	This study (V. Migeot)
2202	Pac1-TAP tor1 :: kanR	Pac1 TAP-tagged tor1 deleted	h+; pac1-TAP-natR; tor1::kanR; ade6-210; ura4-D18; leu1-32; his3-D1	This study
2207	Pac1-TAP sty1 as	Pac1 TAP-tagged Sty 1 Analogue Sensitive	h+; pac1-TAP-natR; sty1 T97A	This study
2217	Pac1-TAP Tor2 L2048S	Pac1 TAP-tagged Tor2 thermo sensitive	h-; pac1-TAP-natR; tor2 L2048S-kanR	This study
2232	Pac1-TAP	Pac1 TAP-tagged	h90; pac1-TAP-natR	This study
2238	Pac1-TAP tsc1 :: kanR	Pac1 TAP-tagged tsc1 deleted	h-; pac1-TAP-natR; tsc1 ::kanR	This study
2245	tor2-L1310P	tor2 constitutively active	h90; tor2 L1310P	(Laboucarié et al., 2017)
2246	rhb1-DA4	rhb1 dominant active	h90; rhb1	(Laboucarié et al., 2017)
2247	Pac1-TAP gcn2 :: hphR	Pac1 TAP-tagged gcn2 deleted	h+; pac1-TAP-natR; gcn2 ::hphR; ade6-210; ura4-D18; leu1-32; his3-D1	This study
2248	Pac1-TAP tsc1 :: kanR gcn2 :: hphR	Pac1 TAP-tagged tsc1 and gcn2 deleted	h-; pac1-TAP-natR; tsc1 ::kanR; gcn2 ::hphR	This study
2256	Pac1-TAP gcn2 :: hphR	Pac1 TAP-tagged gcn2 deleted	h90; pac1-TAP-natR, gcn2 :: hygroR	This study
2261	Pac1-TAP tor2-L1310P	Pac1 TAG-tagged tor2 constitutively active	h90; pac1-TAP;	This study (C. Yague-Sanz)
2262	Pac1-TAP rhb1-DA4	Pac1 TAG-tagged rhb1 dominant active	h90; pac1-TAP;	This study (C. Yague-Sanz)

	Pac1-TAP pREP41 Pac1-T121 S122-Flag	Pac1 TAP-tagged transfected with pDH 955	h90; pac1-TAP-natR; gcn2 ::hphR	This study
	Pac1-TAP pREP41 Pac1-T121A S122A-Flag	Pac1 TAP-tagged transfected with pDH 957	h+; pac1-TAP-natR; ade6-210; ura4-D18; his3-D1	This study
	Pac1-TAP pREP41 Pac1-T121E S122E-Flag	Pac1 TAP-tagged transfected with pDH 956	h+; pac1-TAP-natR; ade6-210; ura4-D18; his3-D1	This study
	Pac1-TAP pREP81 Pac1-T121 S122-HA	Pac1 TAP-tagged transfected with pDH 966	h+; pac1-TAP-natR; ade6-210; ura4-D18; his3-D1	This study
	Pac1-TAP pREP81 Pac1-T121A S122A-HA	Pac1 TAP-tagged transfected with pDH 967	h+; pac1-TAP-natR; ade6-210; ura4-D18; his3-D1	This study
	Pac1-TAP pREP81 Pac1-T121E S122E-HA	Pac1 TAP-tagged transfected with pDH 968	h+; pac1-TAP-natR; ade6-210; ura4-D18; his3-D1	This study
	Pac1-TAP	Pac1 TAP-tagged transfected with pDH 269	h+; pac1-TAP-natR; ade6-210; ura4-D18; his3-D1	This study

Mating

The Pac1-TAP Sty1as, Pac1-TAP Tor2 L2048S and and Pac1-TAP tsc1 :: kanR strains were obtained following a cross between the Pac1-TAP and Sty1as strains, the Pac1-TAP and Tor2 L2048S strains and the Pac1-TAP and tsc1 :: kanR strains respectively.

The crossing is carried out in the presence of two strains of opposite mating type on malt extract-agar at 25°C. The spores produced are digested with 2,5 µL of ß-glucuronidase (Roche 10127060001) in sterile water at 29°C for 6 hours. Once washed with 1% SDS, the *S. pombe* yeasts are cultured on a YES medium and selected on YES nourseothricin, kanamycin or hygromycin depending on the strain. With PCR colony, the mating type of the strains is checked, to make 1 reaction of 100 µL, a master mix is made with 20 µL of 5X buffer, 20 mM of dNTP, 0.7 µL of each primer (Table S1, 727, 728 and 729), one loop of *S. pombe* and the rest of water. To lyse the cells, the program starts with incubation at 100°C for 10 minutes. At the end of the incubation, 0.5 µL of Gotaq enzyme is added. The mutation in Sty1 was verified by Sanger sequencing, after amplification by PCR colony (Table S1, 1424 1425), and TAP-tagged Pac1 is also verified (Table S1, 3622 3623)

Transformation

The Pac1-TAP tor1::kanR, Pac1-TAP, Pac1-TAP Rhb1-DA4, Pac1-TAP Tor2 L1310P, Pac1-TAP gcn2 :: hygroR, Pac1-TAP tsc1 :: kanR gcn2 :: hygroR, pREP41-Pac1-Flag, pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E-Flag S122E, pREP 81, pREP81-Pac1-HA, pREP81-Pac1-T121A S122A-HA et pREP81-Pac1-T121E S122E-HA strains were achieved via transformation (Figure S3).

First, with a expand PCR program, the future insert is produced for the Pac1-TAP tor1::kanR, Pac1-TAP, Pac1-TAP Rhb1-DA4, Pac1-TAP Tor2 L1310P, Pac1-TAP gcn2 :: hygroR, Pac1-TAP tsc1 :: kanR gcn2 :: hygroR strains. The plasmid used (Table S2) initially present in the database, is added to the master mix with 0.8 µL of each primer (Table S3) and 1 µL Expand Taq for a 100 µL reaction. The PCR product is purified according to the QIAquick PCR Purification kit (Qiagen) and the manufacturer's instructions. The strain that will be transformed is pelleted in exponential phase and washed in 1 mL of 0.1M LiAc-TE (1 mM EDTA, 10 mM Tris pH 7.5 and 0.1M lithium acetate) and resuspended in 100 µL of LiAc-TE 0.1M, 2.5 µL of boiled carrier DNA (Sigma salmon sperm DNA) and 15 µL of purified DNA or 2µL of plasmid. After incubation at room temperature for 10 minutes, we incubated our samples again for 30 minutes with 260 µL of LiAc-TE-PEG (40% PEG4000), and finally, we add 43 µL of DMSO before doing a heat shock for 5 minutes at 42°C. To finalize the transformation, yeasts were pelleted and resuspended in YES medium, before plated on a YES medium petri dish or directly on selective medium for strains transformed with a plasmid (EMM-Leu). Strains were checked by PCR colony: *tor1* deletion (Table S1 2321-244), *gcn2* deletion (Table S1 4169-1800), Pac1-TAP tagged (Table S1 3626-244)

pREP41 and pREP81 strains construction.

In the pac1 sequence, threonine 121 and serine 122 have been mutated to alanine or glutamic acid, we have also incorporated the BamH1 and Sal1 restriction sites at the ends of the sequence. The Pac sequence was synthesized by Eurofins Genomics. The synthetic DNAs are amplified, for 1 reaction of 200µL we add to 2µL of Gblock at 1000 ng/µL, 40µL of buffer, 4µL of 10mM dNTP, 1µL of each primer (for pREP41-Pac1-Flag, pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E S122E-Flag strains : Table S1 4108-4101; pREP81-Pac1-HA, pREP81-Pac1-T121A S122A-HA et pREP81-Pac1-T121E S122E-HA strains : Table S1 4108-4168), supplemented with water. The PCR product is purified according to the QIAquick PCR Purification kit (Qiagen) and the manufacturer's instructions.

Ligation of Pac1 in pREP41 and pREP81

The amplified sequences of Pac1 cut by BamH1 and Sal1 are inserted into BamH1-Sal1-cut pREP 81 and pREP41 using T4 DNA ligase (PROMEGA M1808) following the protocol provided by the manufacturer. Finally, 50µL of competent cells (E. coli DH10B) are transfected with 20µL of ligation product. E. coli cells are incubated on ice for 10 minutes and subjected to heat shock at 42°C one minute. After one hour at 37°C in LB Broth Base (Invitrogen). The cells are pelleted and suspended in 100µL of medium and spread on Petry dishes containing LB Broth Base-agar and ampicillin. Insertion of Pac1 sequence is verified by diagnostic restriction, carried out with EcoRV fast digest enzyme and by sanger sequencing.

Restriction reaction

The restriction reactions are carried out the protocol provided by the manufacturer for each enzyme. These reactions were performed with the ThermoFisher Scientific FastDigest enzymes EcoRV (FD0303), BamH1 (FD0054) and Sal1 (FD0644).

Mini-prep

The mini-preps are carried out following the protocol provided by the manufacturer. Using the Sigma GenElute TM Plasmid miniprep kit

Sanger sequencing

Sanger sequencing is carried out with the Mix2Seq Kit, following the manufacturer protocol, and sent to Eurofins Genomics.

Induction of stress

For nitrogen starvation conditions, initially, the Pac1-TAP, Pac1-TAP ste11::kanR, Pac1-TAP tor1::kanR strains are cultured in EMM+AS at 32°C, the Pac1-TAP tsc1 :: KanR, Pac1-TAP gcn2 :: hygroR, Pac1-TAP tsc1 :: kanR gcn2 :: hygroR, Pac1-TAP Tor2 L1310P, Pac1-TAP Rhb1-DA4, prototrophic Pac1-TAP strains are cultured in EMM at 32°C, the pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E S122E-Flag, pREP 81, pREP81-Pac1-HA, pREP81-Pac1-T121A S122A-HA et pREP81-Pac1-T121E S122E-HA strains are cultured in EMM-Leu at 32°C and finally the prototrophic Pac1-TAP was also cultured in minimal medium with 20mM acid glutamic.

Following this, the yeasts were placed in a minimum nitrogen-free medium without supplement (EMM-N) or in a minimal medium with 20mM of proline for a period of 3 hours at 32°C. Under this same nitrogen depletion condition at 32°C, in order to test the Pac1-TAP Sty1as strain, 10 µM of 1NM-PP1 (Sigma 529581-1MG) is added to the EMM-N. To perform the assay in the presence of cAMP, 5 mM cAMP (Sigma A9501) was added to the medium.

For the glucose starvation condition, the Pac1-TAP strain was switched from a yeast extract medium containing 3% glucose to a yeast extract medium containing 0.1% glucose, 3% glycerol and supplemented with adenine, uracil, leucine and histidine (225 mg/L) in incubation at 32°C for 3 hours. Changes of medium involving a nutrient deficiency were carried out by a step of filtration, followed by washing and resuspension with the depleted medium.

Pac1-TAP Tor2 L2048S is cultured in YES and EMM+AS at 25°C and finally incubated at 37°C for 1.5h, 3h, or overnight. The osmotic shock is carried out with equal volume of 2M sorbitol (Sigma S1876) YES medium was added to the culture to reach a final 1M sorbitol concentration, incubated for 3 hours at 32°C. The changes of medium were carried out in the mid-exponential phase of growth.

Bortezomib assay

In order to test the Pac1-TAP Tor2 L2048S strain in bortezomib condition. 10, 30 or 100 µM of bortezomib (SIGMA 5.04314.0001) is added to the EMM + AS medium for 6 hours at 37°C. To perform the assay at 26°C, the Pac1-TAP Tor2 L2048S strain is incubated at 26°C in EMM+AS medium with 30 µM of bortezomib for 4 hours.

Protein extraction

In the exponential growth phase, 10 mL of yeast are pelleted at 4000 rpm for 4 minutes and washed in NP40 buffer (1 M sorbitol, 50 mM NaCl, 10 mM tris HCl pH 7.4, 5 mM MgCl2, 1

mM CaCl2, NP40 0.75%, supplemented with water). Once the pellet is suspended in NP40 buffer, the protein extraction is carried out with zirconium beads in FastPrep 4 cycles at 5.5 m/s for 20 seconds followed by a one minute rest. The beads are washed once with NP40 buffer. The washed product is centrifuged for 10 minutes at maximum speed, to recover the proteins in suspension.

Western blot, transfer and membrane revelation

To perform the labelling of total proteins in the samples, we use 16 µL of protein extract, to which is added 1µL of Cy5 (10X) and 3µL of buffer, for a 10-minute incubation on ice. Before loading 15 µL of Cy5-labelled protein extracts onto Mini-PROTEAN TGX Precast Gels 4-15% Bio-Rad, the extracts are heated at 100°C for 4 minutes with 6.6 µL of Beta-mercapto (10% in buffer 4x). The migration schedule performed is 10 minutes at 100 volts followed by 35 minutes at 140 volts. Transfer of the gel is carried out using the Bio-Rad Trans-Blot Turbo Transfer System kit, with a 7-minute program suitable for proteins with sizes between 5 and 150 kDa (MIXED MW program). The membrane is blocked with milk (Skin milk powder) (Sigma-Aldrich) (5% PBS Tween) for one hour, with agitation at room temperature. Immunoblotting is performed with an anti-TAP antibody, PAP (*Peroxidase anti-peroxidase*. sigma P1291).

For strains containing exogenous Pac1 tagged with a flag or a HA, the membrane is incubated at RT with the first antibody (anti-HA Sigma H6908; or anti-Flag Sigma F3165). After 1 hour of incubation, the membrane is washed 3 times with PBS tween, before being exposed to the second antibody (Anti-mouse: IgG peroxidase GE NA931V, anti-rabbit: IgG peroxidase GE NA934V, respectively) during 1 hours at RT. The membrane is placed a second time, twice in PBS tween and once in PBS. Finally, we reveal the membrane, on the Imagequant 800 machine, in multiplex fluorescence and chemiluminescence with the Western Lightning® Plus-ECL (NEL104001EA), in automatic detection.

RNA extraction

In the exponential growth phase, 10 mL of yeast are pelleted at 4000 rpm for 4 minutes and washed in DEPC H_2O, and stored at -80°C. The pellet is resuspended in 750 µL of TES buffer (500 µL de Tris HCl pH 7,5 1M, 1000 µL EDTA pH 8 0,5M, 1500 µL SDS 10% supplemented with d'H2O DEPC) and in equivalent volume of phenol (Sigma-Aldrich P1944-400ML). Each sample is incubated at 65°C for one hour and vortexed every 10 minutes for 10 seconds at 1300 rpm. The samples are then incubated for 1 minute on ice and pelleted at 14 000 rpm for 15 minutes.

The aqueous phase is recovered and supplemented with the same volume of phenol (Sigma Aldrich 77619-500ML). The samples are centrifuged at 14 000 rpm for 5 minutes. Once again, the aqueous phase is recovered and supplemented with the same volume of phenol (Supelco 25666-500ML) and centrifuged at 14 000 rpm for 5 minutes. Finally, 500 µL of the aqueous phase is taken to which 1500 µL of 100% ethanol and 50 µL of sodium acetate buffer solution (SIGMA S17899-10ML) are added. The samples are incubated for 1 hour at -80°C and pelleted for 10 minutes at 14,000 rpm. The RNA pellets are washed with 70% ethanol and dried at RT to remove all traces of ethanol. These are detached by heat with incubation at 65°C for 5 minutes and resuspended in DEPC H_2O.

RtqPCR

Complementary DNA (cDNA) are synthesized with an RT program. In order to carry out RTqPCR, for each condition we make a master mix composed of 5µL of cDNA, 12.5 µL of syber green (BIO-RAD), 1 µL of each 10 µM primer and supplemented with water. The primers (Table S1) used to amplify Pac1: 3626-3627, Pac1-HA 4216-4217, Pac1-TAP: 1782-4216, Actin: 739-740, Mfs2: 3622-3623. RNA quantification is carried out on actin.

Protease and phosphatase inhibitors

During the preparation of the cell pellets, the yeasts were resuspended in NP40 buffer PhosSTOP (Roche 4906837001) and/or in NP40 buffer cOmplet (Roche 5056489001) following the manufacturer's instructions. Protein extraction is performed with NP40 supplemented with inhibitors.

Phosphatase

The protein samples were treated with Lambda phosphatase (Bio labs - P0753S) following the manufacturer's instructions.

Drop assay

Pac1-TAP Tor2 L2048S was cultured in YES at 25°C, Pac1-TAP pREP41 were cultured in EMM-Leu with or without thiamine (Sigma T3902) at 32°. Once the cells are in exponential phase, by centrifugation (4 minutes at 4000 rpm) and dilution in the medium, the OD595 of the cells is reset to 1. This allows serial dilutions 5x to be made, to finally place the drops on a YES medium petri dish and incubate at 25°C or 37°C overnight for the Pac1-TAP Tor2 L2048S strain. For the pREP41-Pac1 -Flag, pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E S122E-Flag and Pac1-TAP strain, we performed the drop assay on EMM-Leu with thiamine medium or EMM-Leu medium Petri dish and incubate at 32°C.

Growth assay

After an overnight preculture at 32°C, the cells are diluted to OD595nm = 0,1. In a 96 wells plates (Greiner Bio-One 655180), 100µL of yeast are placed, some wells are filled with 150µL of EMM-Leu or EMM-Leu thiamine (Sigma T3902) medium to have blanks. Peripheral wells are filled with water. The 96 wells plate is incubated in the BioTek Epoch 2 microplate spectrophotometer at 32°C with linear shaking at 1096cpm for 52 hours. The OD is measured every 20 minutes.

Annexes

1. Abbreviations

°C	Celsius degree
AS	Analogue sensitive
ATP	Adenosine triphosphate
Btz	Bortezomib
bZIP	Basic leucine zipper
cAMP	Cyclic adenosine monophosphate
Cas9	CRISPR associated protein 9
CDK	Cylcin-dependent kinase
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
crRNA	CRISPR RNA
CTD	C-terminal domain
Cy5	Cyanine 5
DNA	Deoxyribonucleic acid
EMM	Edinburgh minimal media
EMM - N	Edinburgh minimal media free-nitrogen
EMM + AS	Edinburgh minimal media all supplements
EMM-Leu	Edinburgh minimal media without leucine
EMMG	Edinburgh minimal media with glutamic acid
EMMP	Edinburgh minimal media with proline
GAP	GTPase activating protein
GATOR	GAP activity toward Rag GTPases1
GDP	Guanosine diphosphate
GEF	GTP exchange factor
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
h	Hour
h-	Minus
h+	Plus
h90	Homothalic strain
HMG	High mobility group
kb	Kilobase
kDA	Kilo Dalton
L	Liter

MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
mG	Miligramme
miRNA	Micro RNA
mM	Milimolaire
OD	Optical density
PCR	Polymerase Chain reaction
pH	Potentiel of hydrogen
PKA	protein kinase A
pREP	Replicative plasmid
RNA	Ribonucleic acid
RNA pol II	RNA polymerase II
rpm	Revolution per minute
S122	Serine 122
T121	Threonine 121
TOR	Target of rapamycin
TORC1	Target of rapamycin complex 1
TORC2	Target of rapamycin complex 2
TR boxe	T-rich boxe
tRNA	Transfert RNA
Ts	Termosenstive strain
TSC	Tuberous sclerosis
TSS	Transcription start sites
V	Volt
YES	Yeast extract with supplements
µL	Microliter
µM	Micromolaire

2. Additional data

Table S1. List of primers used in this Master thesis.

Database Number	Description	Sequence
244	NatR-KanR check reverse	CGGATGTGATGTGAGAACTGTAT CCTAGC
727	MT1	AGAAGAGAGAGTAGTTGAAG
728	Mating P	ACGGTAGTCATCGGTCTTCC
729	Mating M	TACGTTCAGTAGACGTAGTG
739	Actine forward	CCACTATGTATCCCGGTATTGC
740	Actine reverse	CAATCTTGACCTTCATGGAGCT
1424	sty1 G forward	CTTAATCACAGCTACATACCTCC
1425	sty1 G reverse	TTAGAAAAGGAGTGGCGAG
1782	Tap-tag reverse	GCTGAGACGGCTATGAAATTC
1899	hph reverse	GCATCAGCTCATCGAGAGCCT
2319	tor1 deletion forward	GGAAGAATTGAACACCGCGACTAT TAGAAAGTCTATCGTTTCACTCGCT CTCTTTGATTC – CGGATCCCCGGGT TAATTAA
2320	tor1 deletion reverse	GACATAAATTAATAACAACACGAAA AAAATTATCATAATCTCAAAAAACA GAAAACATCA – GAATTCGAGCTCGT TTAAAC
2321	tor1 deletion check forward	TGTCGGGAAATCAACCAAAC
2377	tsc1 deletion check forward	TTGCCCTCTAACTTCCTTTG
3622	msf2 ORF F	GCAATGATTCAGGCAGCTACTC
3623	msf2 ORF R	GTAGTTAATGATGTACACCACAGTC
3626	pac1 ORF forward	CACTTGTCCTCAGTTATTCTGC
3627	pac1 ORF reverse	GTCTCTTCTTGTCCATCCAATATC
4063	pac1 C-term tag forward	CAAATCAAAAAGACGCGGGCTCTAGG GCAGCTATGCAAGCACTCGAAGTCCTT GCAAAAGATTACTCTAAGTTTGCCCGT - CGGATCCCCGGGTTAATTAA
4064	pac1 C-term tag reverse	AGGACCAAACAAAAGATAGATTGACT GTGACAGTAAGACCTTTTAACGATCGA AACCGTGACGAATTAATTCATCGTATA - GAATTCGAGCTCGTTTAAAC
4101	amplification Gblock Pac1 reverse	CGCGGATCCTTAACGCTTAT
4108	amplification Gblock Pac1 Forward	ACGCGTCGACATGGGACGGT
4168	pac1 HA-tagged reverse	CGCGGATCCTTAAGCGTAATCTGGAACA TCGTATGGGTATCCACCTCCACGGGCAA ACTTAGAGTAAT

4169	gcn2 deletion check forward	CAAACAATGAAGCGCTTTGC
4170	gcn2 deletion forward	ACAGTTTAATTGTGAATAAATATATTTGC TTCCTATTAAACTAGTAAATTATTGGCAA CAATAGTTGGTAAAAGCTGTTCCGGATCC CCGGGTTAATTAA
4171	gcn2 deletion reverse	CATTTAAAGTAATTTATAGCAAAATTTTT TCTATTTAATTAAAAGAGTAATGAAAAT TAAATGATTAATTAGTTCAAAATGAATTC GAGCTCGTTTAAAC
4176	Amplification Gblock pac1-CRISPR F	AAAGGATGATGTGAATTTGGTTA
4177	Amplification Gblock pac1-CRISPR R	GCCTTTCATTATGGATATCCAAC
4177	pac1 ORF forward	GGACGGTTTAAGAGGCATCA
4181	sgRNA pac1	ATAGTTGCTGTTGCCAAAAAACATAA CCTGTACCGAAGAA – AAATAATGAAC CAACATCTG - gttttagagctagaaatagcaag
4216	pac1 ORF forward	CTAGGGCAGCTATGCAAGC
4217	HA-tag reverse	AGCGTAATCTGGAACATCGT

Table S2. List of plasmids used in this Master thesis.

Numero data base	Promotor	Insert
pDH 94	pREP-41	/
pDH 190	pFA6A	kan-MX6
pDH 269	pREP-81	/
pDH 285	pFA6A	Pac1-TAP 4X
pDH 371	pFA6A	HphR
pDH 955	pREP-41	pac1-Flag
pDH 956	pREP-41	pac1-Flag T121E S122E
pDH 957	pREP-41	pac1-Flag T121A S122A
pDH 966	pREP-81	pac1-HA
pDH 967	pREP-81	pac1-HA T121A S122A
pDH 968	pREP-81	pac1-HA T121E S122E

Table S3. List of transformed strains used in this Master thesis.

data base number	Name	Plasmid amplified	Primers	Strain transformed		Verification primers
2202	Pac1-TAP tor1 :: kanR	pDH 190	2319-2320	2114	Pac1-TAP	2321-244
2232	Pac1-TAP	pDH 285	4063-4064	568	Wild-type	3626-244
2247	Pac1-TAP gcn2 :: hphR	pDH 371	4170-4171	2114	Pac1-TAP	4169-189

2248	Pac1-TAP tsc1 :: kanR gcn2 :: hphR	pDH 371	4170-4171	2238	Pac1-TAP tsc1 :: kanR	4169-189
2256	Pac1-TAP gcn2 :: hphR	pDH 371	4170-4171	2232	Pac1-TAP	4169-189
2261	Pac1-TAP tor2-L1210P	pDH 285	4063-4064	2245	tor2-L1310P	3626-244
2262	Pac1-TAP rhb1-DA4	pDH 285	4063-4064	2246	rhb1-DA4	3626-244
	Pac1-TAP pREP41 Pac1-T121 S122-Flag	pDH 955	/	2114	Pac1-TAP	/
	Pac1-TAP pREP41 Pac1-T121A S122A-Flag	pDH 957	/	2114	Pac1-TAP	/
	Pac1-TAP pREP41 Pac1-T121E S122E-Flag	pDH 956	/	2114	Pac1-TAP	/
	Pac1-TAP pREP81 Pac1-T121 S122-HA	pDH 966	/	2114	Pac1-TAP	/
	Pac1-TAP pREP81 Pac1-T121A S122A-HA	pDH 967	/	2114	Pac1-TAP	/
	Pac1-TAP pREP81 Pac1-T121E S122E-HA	pDH 968	/	2114	Pac1-TAP	/
	Pac1-TAP pREP81	pDH 265	/	2114	Pac1-TAP	/

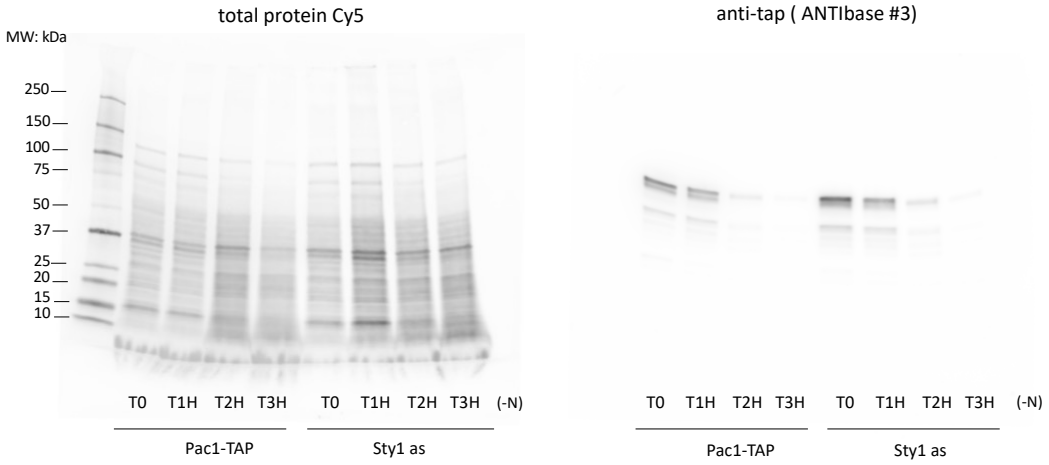

Figure S1. Western blot analysis of TAP-tagged Pac1 over three hours of nitrogen starvation (EMM-N) with 10µM of 1NM-PP1 in wild-type and Sty1 AS strains.

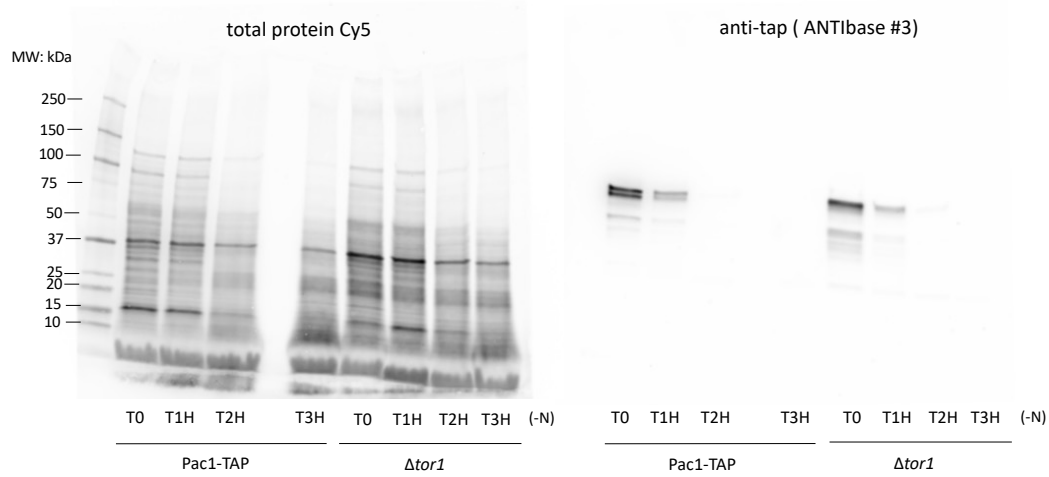

Figure S2. Western blot analysis of TAP-tagged Pac1 over three hours of nitrogen starvation (EMM-N) in wild-type and Δ*tor1* strains.

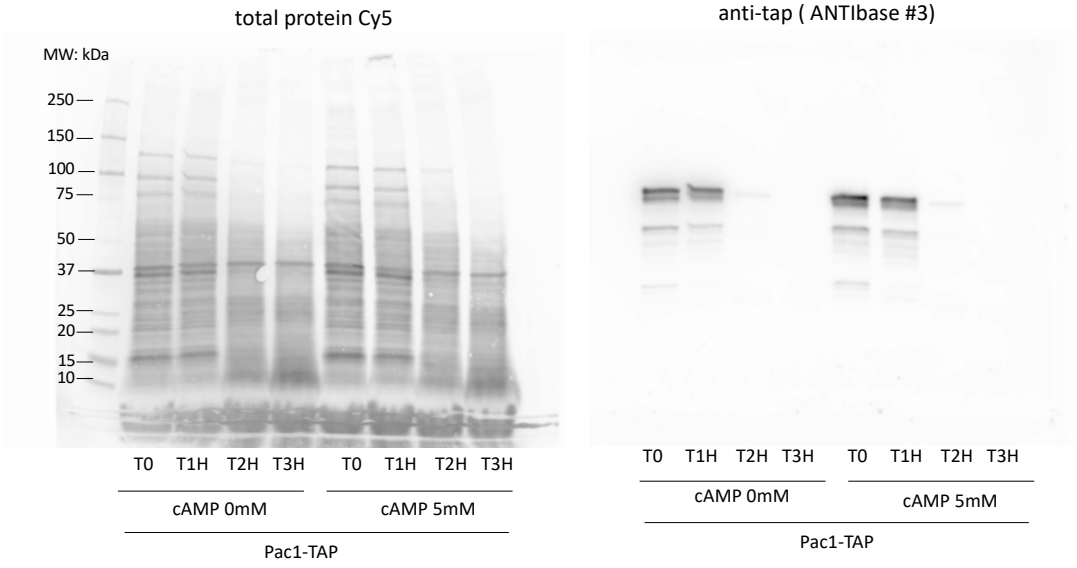

Figure S3. Western blot analysis of TAP-tagged Pac1 over three hours of nitrogen starvation (EMM-N) supplemented or not with 5 mM cAMP in wild-type strain.

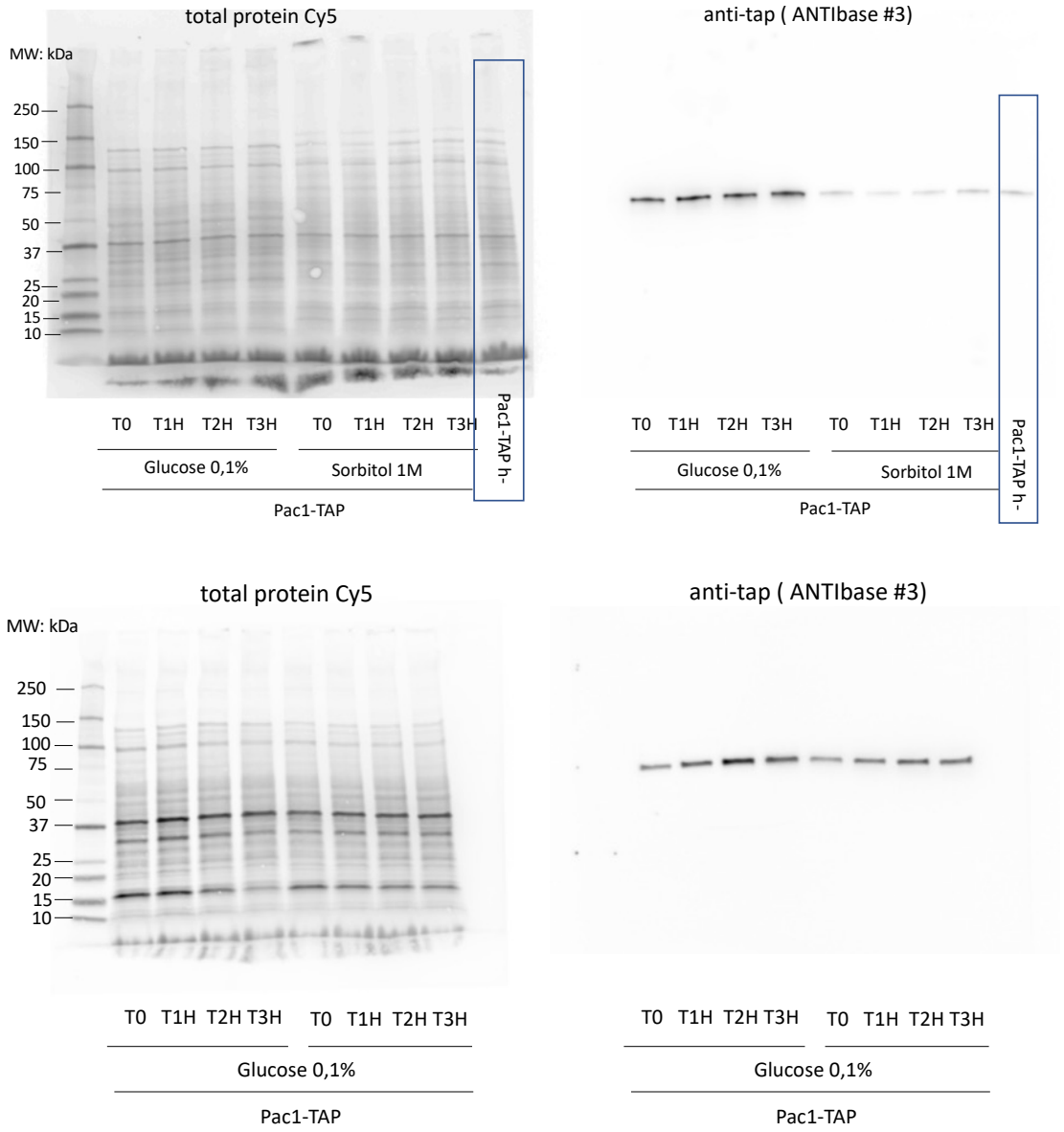

Figure S4. Western blot analysis of TAP-tagged Pac1 over three hours of glucose starvation (YES 0.1% glucose) or in rich medium with osmotic stress induction (1M sorbitol) in wild-type strain.

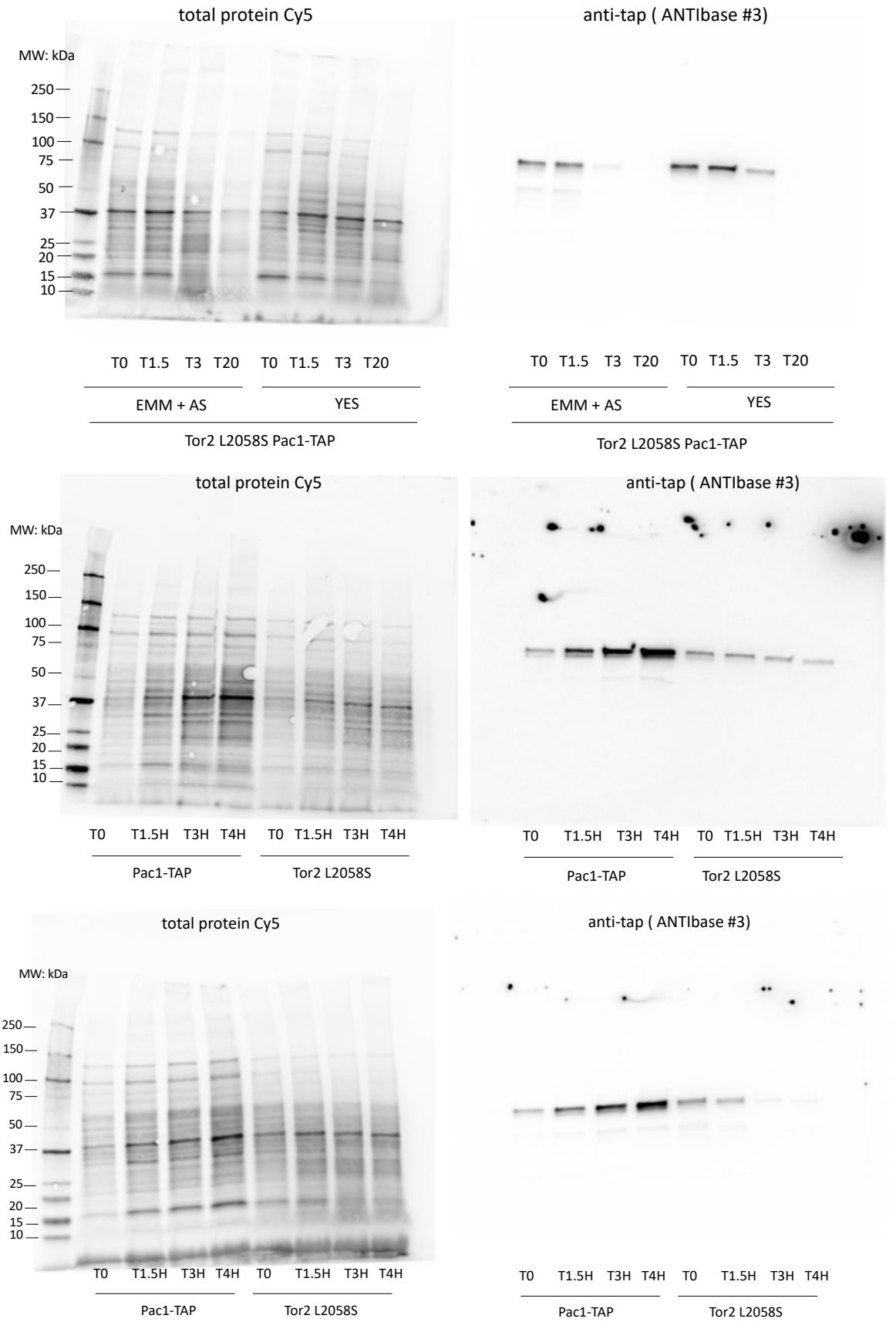

Figure S5. Western blot analysis of TAP-tagged Pac1 in the Tor2 L2048S Pac1-TAP strain, in EMM+AS or in YES over a period of 4 hours or 20 hours after a temperature shift at 37°C.

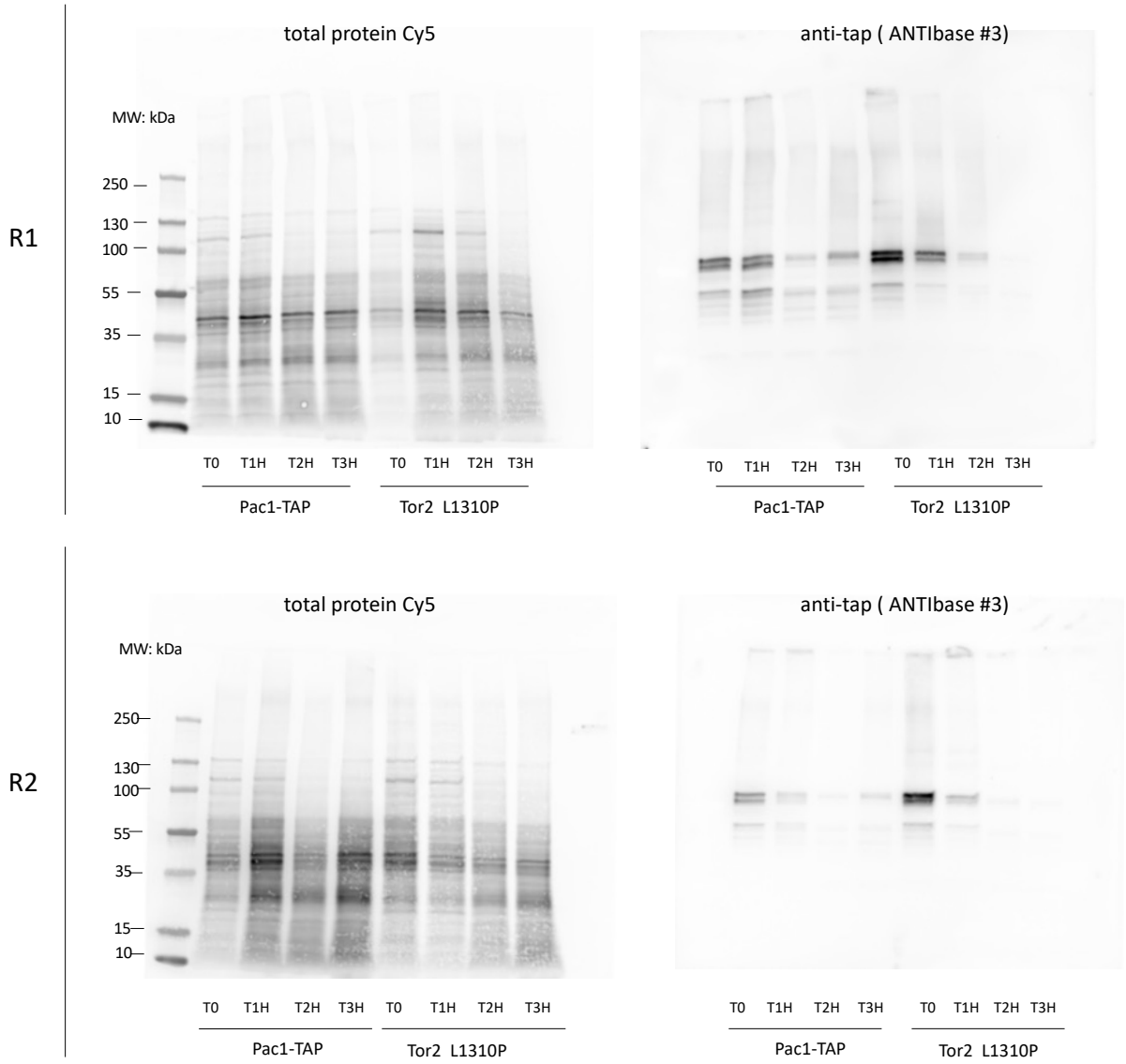

Figure S6. Western blot analysis of TAP-tagged Pac1 over a period of 3 hours in EMMP in a wild-type and Pac1-TAP Tor2 L1310P strains.

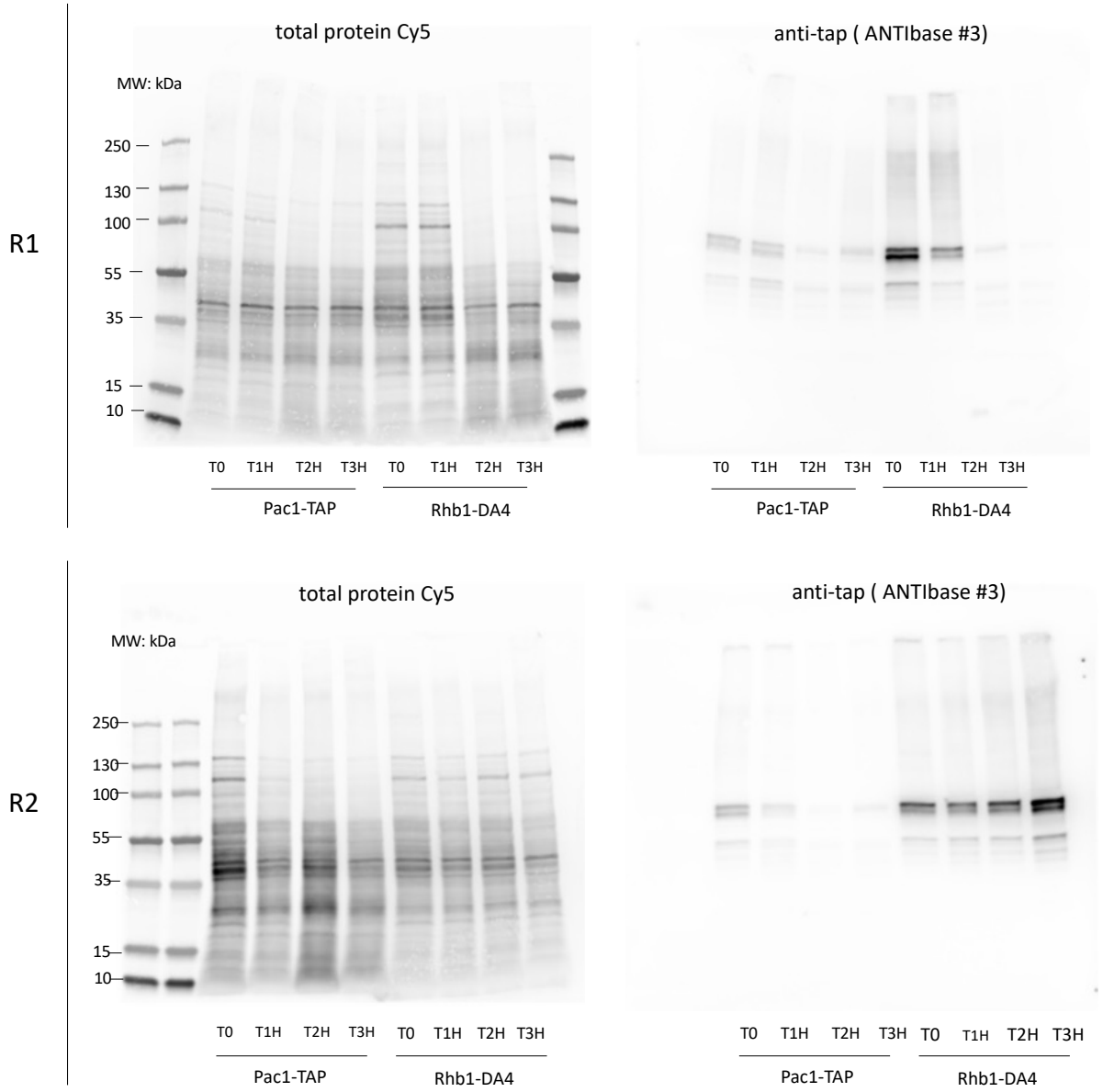

Figure S7. Western blot analysis of TAP-tagged Pac1 over a period of 3 hours in EMMP in a wild-type and Pac1-TAP Rhb1-DA4 strains.

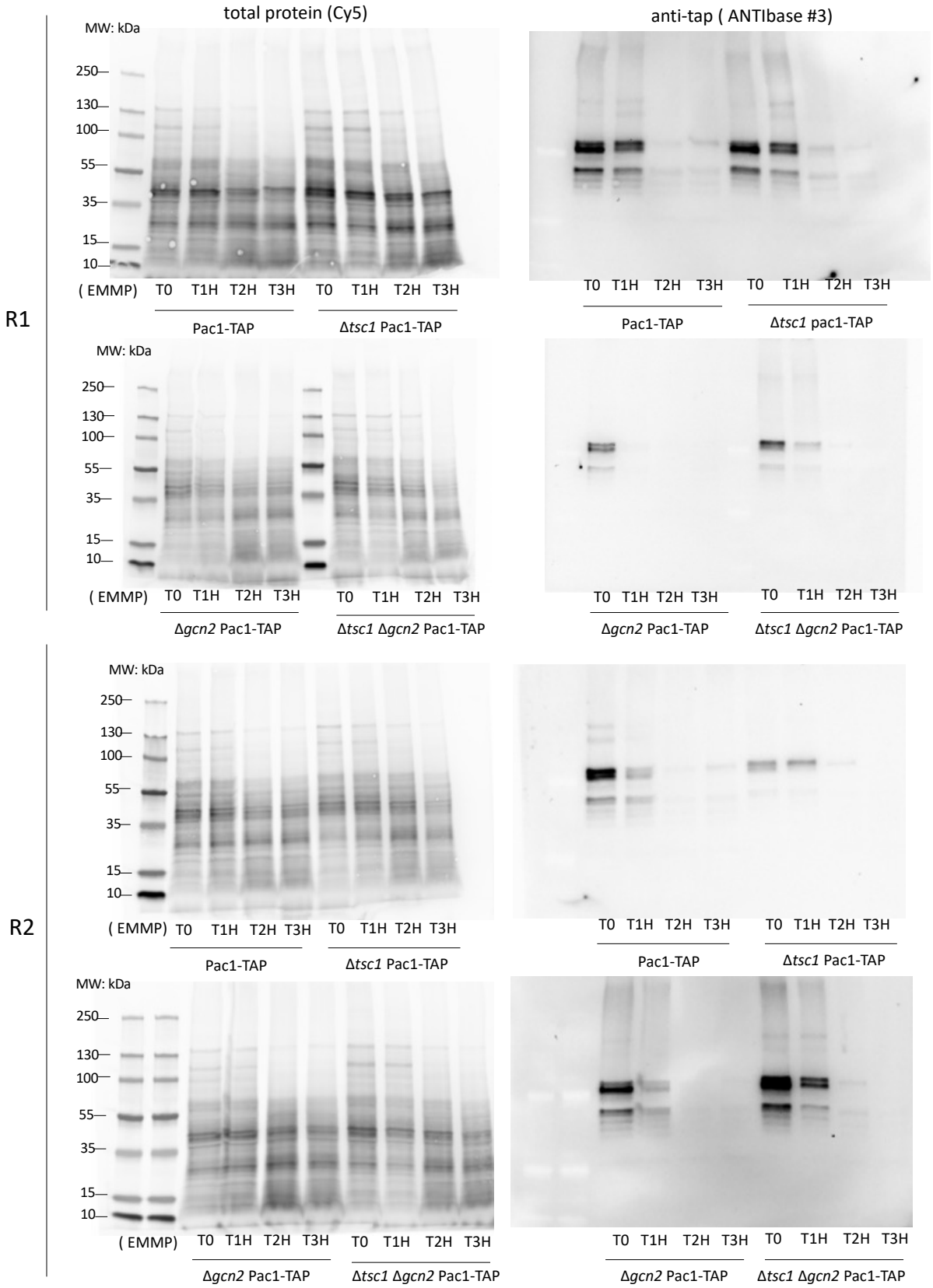

Figure S8. Western blot analysis of TAP-tagged Pac1 over a period of 3 hours in EMMP in a wild-type, Δ*tsc1*, Δ*gcn2* and Δ*tsc1* Δ*gcn2* strains.

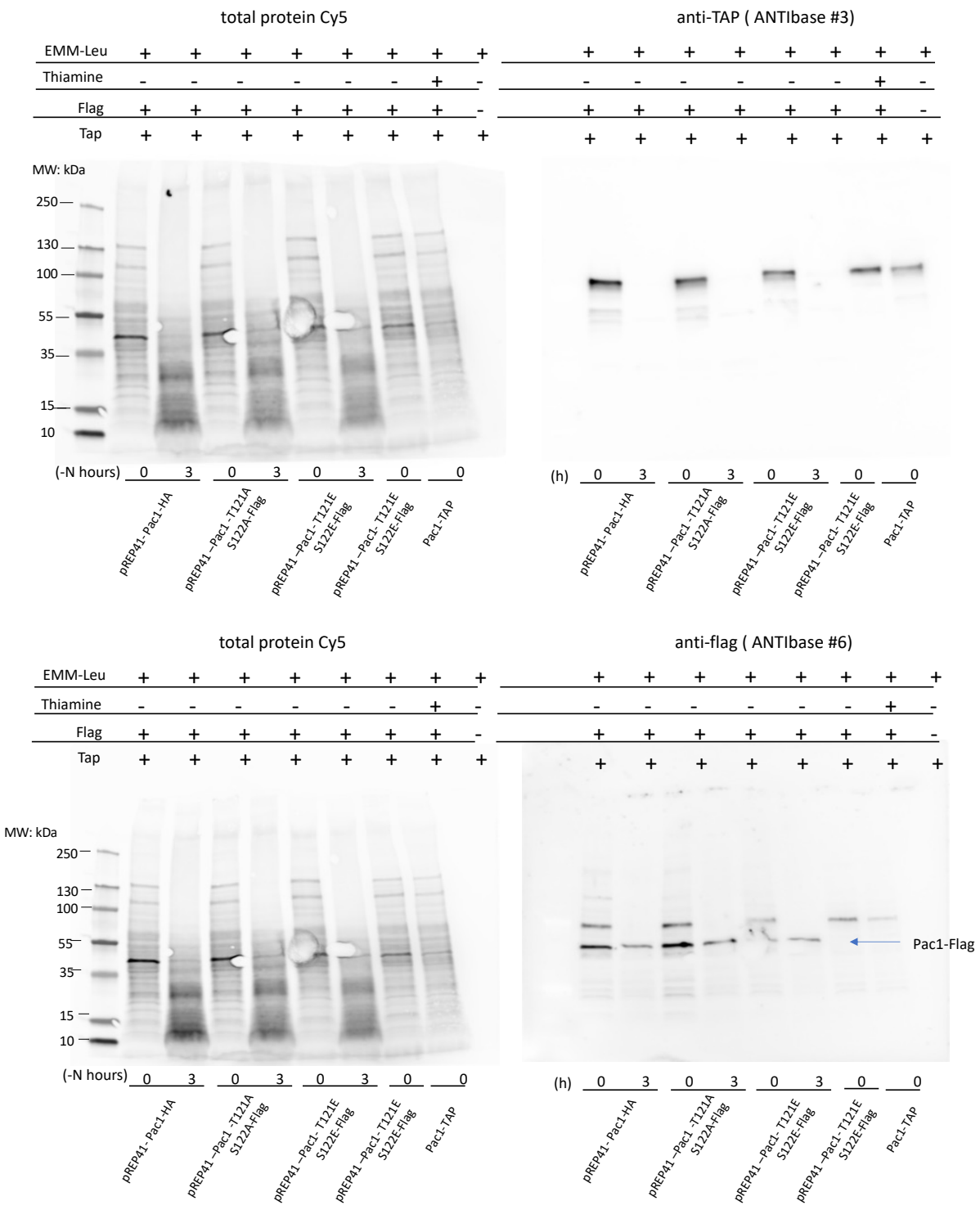

Figure S9. Western blot analysis of TAP-tagged Pac1 and Flag-tagged Pac1 over a period of 3 hours in EMM-N in a wild-type, pREP41-Pac1-Flag, pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E S122E-Flag strains.

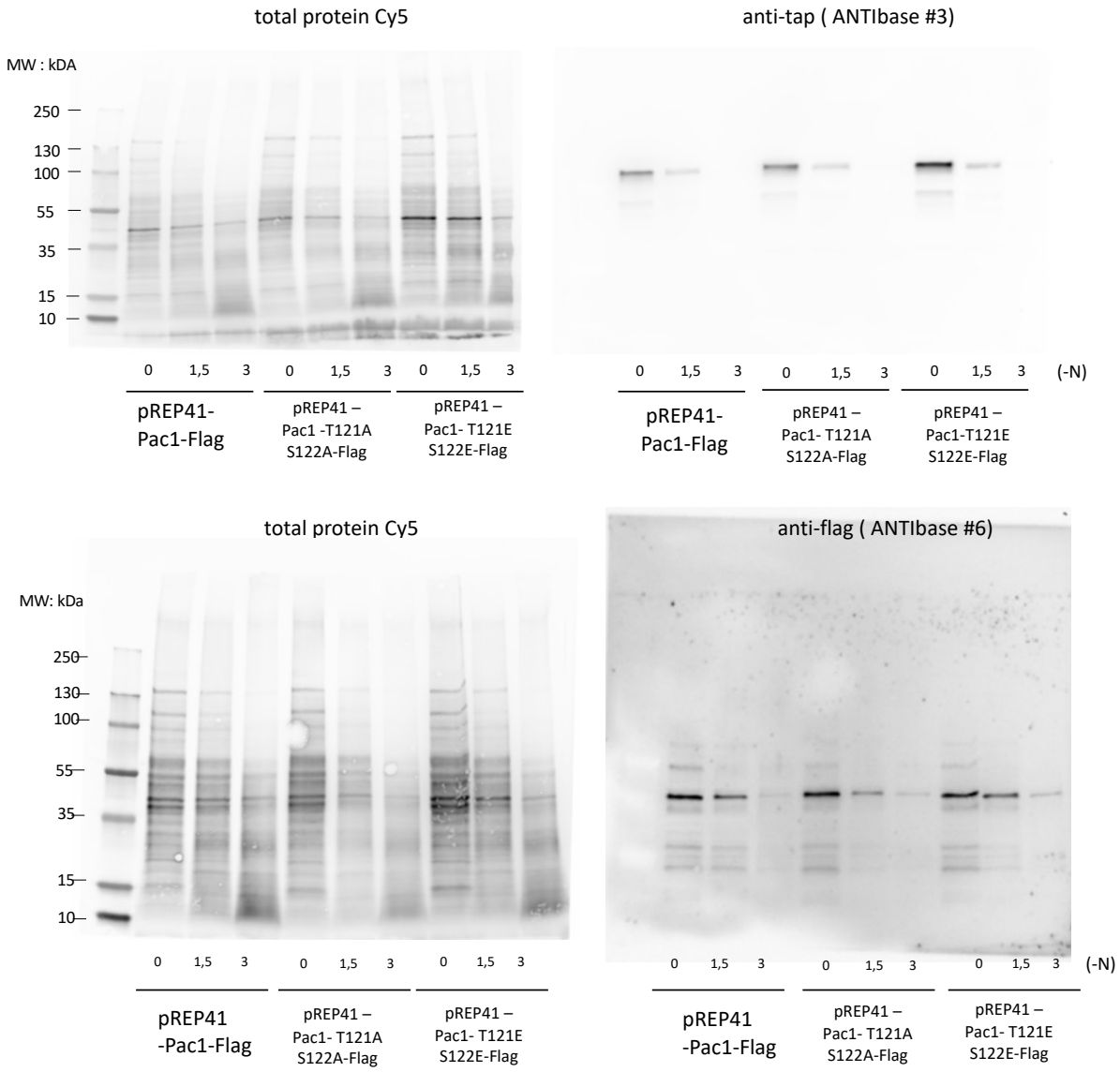

Figure S10. Western blot analysis of TAP-tagged Pac1 and Flag-tagged Pac1 over a period of 3 hours in EMM-N in a wild-type, pREP41-Pac1-Flag, pREP41-Pac1-T121A S122A-Flag, pREP41-Pac1-T121E S122E-Flag strains.

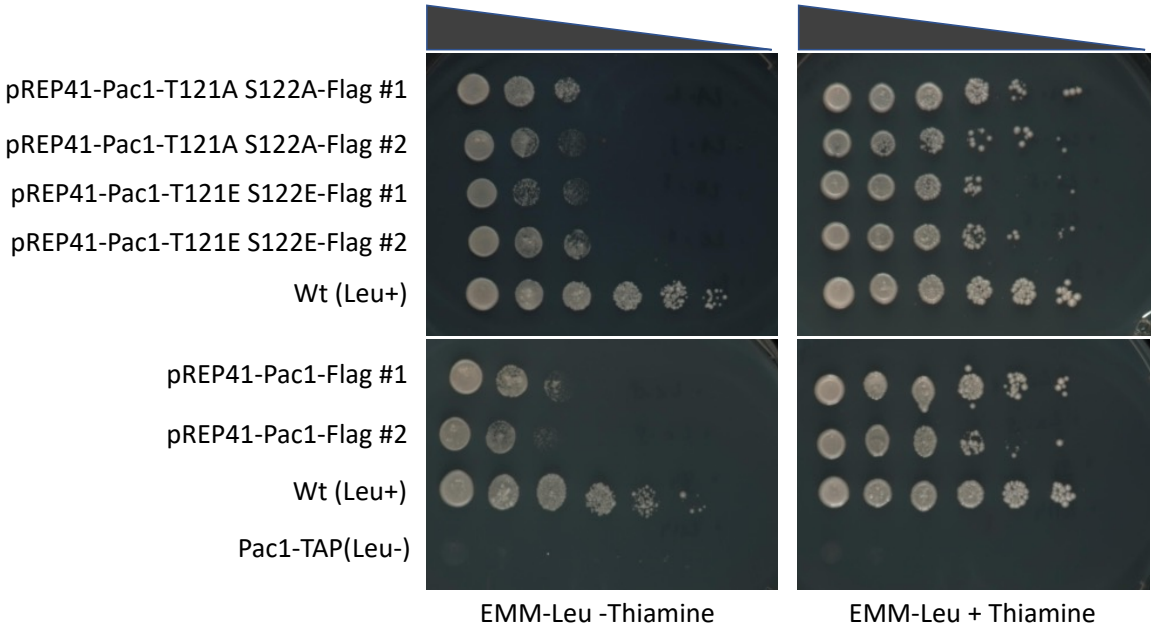

Figure S11. Drop assay of pREP41-Pac1-Flag, pREP41-Pac1 T121A S122A-Flag, pREP41-Pac1 T121E S122E-Flag, wild-type (Leu+) and Pac1-TAP (Leu-) strains incubated in EMM-leu with or without thiamine at 32°C for three days.

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