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Screening for inhibitors of Elongator, a central actor of cancerous initiation and development

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**UNIVERSITÉ
DE NAMUR**

Faculté des Sciences

Screening for inhibitors of Elongator, a central actor of cancerous initiation and development

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

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Janvier 2021

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Screening for inhibitors of Elongator, a central actor of cancerous initiation and development

MARCHAND Florian

Abstract

The role of tRNAs in translation makes them essential for every living organism. The first tRNA modification was discovered in 1965 and since then, the number of modifications has increased to reach more than 100 different chemistries. Our research project focus on a doubly modified uridine positioned at the wobble position of three tRNAs within the anticodon stem loop. This complex double modification is the 5-methoxycarbonylmethyl-2-thiouridine at position 34 ($mcm^5s^2U_{34}$). A plethora of proteins is required for the synthesis of $mcm^5s^2U_{34}$. Among them, Elongator is a conserved, 6-subunits complex (Elp1-6), which takes part in the synthesis of the cm^5 moiety of the modification. Recent studies have highlighted the critical role of Elongator in several diseases, mainly linked with brain development. However, Elongator is also required for tumorigenesis, especially in the case of melanoma, breast cancers and Wnt-dependent tumour initiation. Our research aims at performing a molecular screen to isolate compounds acting as Elongator inhibitors with the hope to treat the associated cancers. The screening strategy relies on the ability of the *Kluyveromyces lactis* toxin, zymocin, to cleave $mcm^5s^2U_{34}$ modified tRNAs, which led to cell death, while unmodified tRNAs are unaffected. Our goal is to construct a strain of *Schizosaccharomyces pombe* expressing the human Elongator complex together with the zymocin catalytic subunit under the control of an inducible promoter. Compounds providing resistance to induction of the toxin will be further characterized as they likely target the synthesis of the modification.

Mémoire de master 120 en biochimie et biologie moléculaire et cellulaire

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Criblage à la recherche d'inhibiteurs d'Elongator, un acteur central dans l'initiation et le développement cancéreux

MARCHAND Florian

Résumé

Le rôle joué par les ARNt lors de la traduction fait d'eux des acteurs essentiels pour tout organisme vivant. C'est en 1965 que les premières modifications ont été découvertes et leur nombre a depuis dépassé la centaine de chimie différente. Notre projet de recherche s'intéresse plus particulièrement à la double modification de l'uridine en position wobble sur la boucle de l'anticodon de trois ARNts. Cette double modification nommée 5-méthoxycarbonylméthyl-2-thiouridine est présente en position 34 ($mcm^5s^2U_{34}$). Une pléthore de protéines est requise pour la synthèse de $mcm^5s^2U_{34}$. Le complexe Elongator, très conservé et composé de 6 sous-unités (Elp1-6) catalyse la synthèse de la chaîne cm^5 . De récentes études ont révélé le lien entre Elongator et plusieurs maladies, la plupart touchant le développement cérébral. Cependant, Elongator est aussi requis pour l'initiation et la progression de divers cancers comme les cancers du sein, les mélanomes ainsi que l'initiation des tumeurs dépendantes de Wnt. Notre projet a pour but l'identification de composés chimiques capables d'inhiber Elongator dans l'espoir de bloquer la progression de la maladie. La stratégie de criblage repose sur l'expression d'une toxine sécrétée naturellement par *Kluyveromyces lactis*, la zymocin, une endonucléase qui cible les ARNts possédant la modification $mcm^5s^2U_{34}$, induisant la mort cellulaire. A l'opposé, les ARNts non modifiés ne sont pas affectés par la toxine. En conclusion, la finalité de notre projet est de réaliser un crible sur une souche de *Schizosaccharomyces pombe* exprimant la version humaine d'Elongator ainsi que la sous-unité catalytique de la zymocin sous le contrôle d'un promoteur inductible. Les composés induisant une résistance à l'expression de la toxine seront caractérisés par la suite pour évaluer s'ils ciblent la synthèse de la modification.

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Janvier 2021

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Introduction

Since the discovery of the first modified nucleoside, pseudouridine, in 1957, about 170 modifications were identified (Grosjean, 2015; Ranjan and Leidel, 2019). Even if the role of these RNA modifications is unclear, some of them present on tRNAs were shown to be important to translation regulation. Among them is the double 5-methoxycarbonylmethyl-2-thiouridine ($mcm^5s^2U_{34}$) at the wobble nucleoside of the anticodon stem loop of $tRNA_{UUU}^{Glu}$, $tRNA_{UUU}^{Lys}$ and $tRNA_{UUU}^{Gln}$. The mcm^5s^2 double modification is synthesized by a set of proteins including the Elongator complex (Kolaj-Robin and Séraphin, 2017).

Elongator is a six subunits structure discovered in yeast by the Svejstrup lab in 1999. The complex was first described as being associated to the hyper-phosphorylated RNA polymerase II (RNAPII). Its hypothetical role was to switch the RNAPII from initiation towards elongation and to control transcription elongation. It was also thought that Elongator could methylate DNA or histones because the catalytic subunit Elp3 possesses two specific domains, an rSAM domain (radical S-adenosyl methionine binding) and a HAT domain (Histone acetyltransferase) now called KAT (for Lysine acetyltransferase). Currently, it seems more likely that Elongator uses both domains to modify the tRNA wobble uridine (U) to 5-carboxymethyluridine (cm^5U) (Huang et al., 2005; Kolaj-Robin and Séraphin, 2017; Otero et al., 1999).

Recent studies have shown that Elongator is linked to multiple human diseases including cancers. The teams of Close and Chariot have discovered that Wnt-dependent tumour initiation decreased when the Elp3 subunit is inactivated. This effect results from the decrease of SOX9 translation, an important actor of cancer stemness maintenance. This study brings to the fore the possibility of targeting the Elongator complex as a new therapeutic path (Ladang et al., 2015).

1. History of Elongator discovery

1.1. First description of Elongator

In 1999, the Svejstrup lab was focused on the comprehension of mechanisms that rule the elongation step of transcription. At this time, it was known that the RNA polymerase II (RNAPII) was the central actor helped by several complexes as for example, the Mediator complex or the general transcription factors TBP, TFIIA, TFIIB, TFIIE, TFIIIF, and TFIIH (Otero et al., 1999; Pokholok et al., 2002). The regulation of RNAPII was based on the phosphorylation state of the carboxy-terminal domain (CTD), which was hypo-phosphorylated at initiation and termination but hyper-phosphorylated during the elongation step. To study this mechanism, the Svejstrup lab purified elongating RNAPII from *Saccharomyces cerevisiae* chromatin and in the same time isolated for the first time a new protein complex named Elongator. This complex, composed of three proteins of 150, 90 and 60 kDa, had a stable interaction with the hyper-phosphorylated CTD of RNAPII. They identified a scaffold protein from this complex, which they called Elp1 for Elongating protein1 (150 kDa). They showed that the mutation of *ELP1* delayed the time of adaptation for the *ELP1* Δ strain when growth conditions were changing and resulted in sensitivity to salt. They concluded that this complex is part of the RNAPII holoenzyme binding to hyper-phosphorylated CTD with a role in transcript elongation (Otero et al., 1999).

The same year, the Svejstrup lab reported that Elp3 is a histone acetyltransferase subunit of Elongator and a conserved member of the GNAT (Gcn5-related N-acetyltransferase) protein family. The deletion of this gene leads to a slow growth adaptation, a slow gene activation and a temperature sensitivity similar to *ELP1* deletion. *In vitro*, Elongator was able to acetylate the

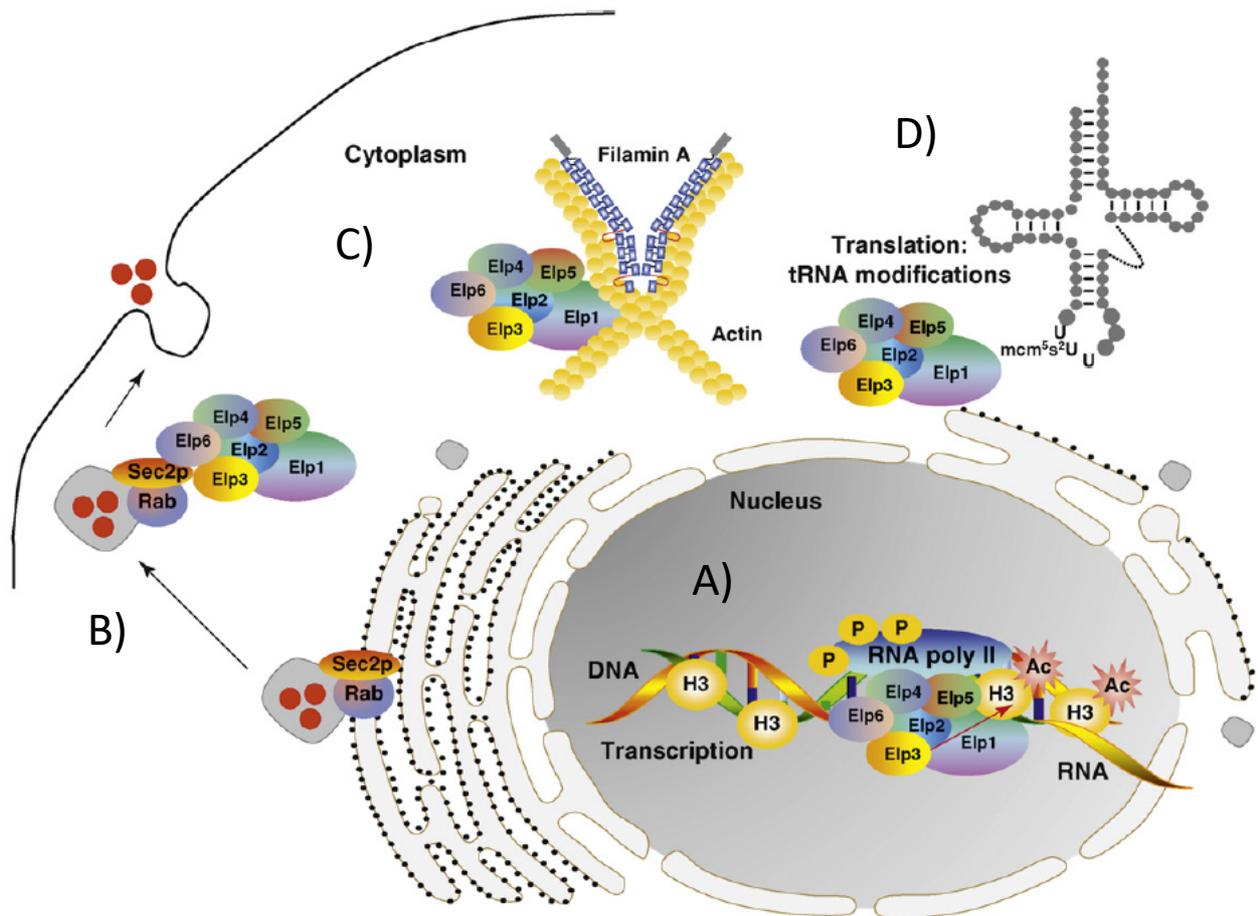


Figure 1. Biological roles of Elongator. A) The regulation of transcription through the acetylation of histones H3 and H4 (not represented on figure). B) The regulation of exocytosis through the localization control of Sec2 which regulates post-Golgi secretion vesicles with Sec4. C) The regulation of cell migration through tubulin acetylation. D) The regulation of translation through the mcm⁵s² modification of uridine at position 34 of tRNA^{Glu}_{UUC}, tRNA^{Lys}_{UUU} and tRNA^{Gln}_{UUG}. From (Nguyen et al., 2009).

four histones. Such ability could serve the RNAPII to process through the chromatin template and to facilitate the transcription (Wittschieben et al., 1999).

In 2001, the Svejstrup lab showed that Elongator is divided in two discrete sub-complexes forming the holo-enzyme. The second sub-complex is composed of three new polypeptides, Elp4 (50kDa), Elp5 (35kDa) and Elp6 (30kDa). Moreover, the deletion of the genes encoding these proteins showed the same phenotypes that the three first subunits, Elp1, Elp2 and Elp3. The combined deletion of several *ELP* genes did not show additional phenotypes. At this time, three human homologues were identified for Elp1, Elp3 and Elp4. They concluded that the functional complex is conserved and composed of 6 subunits with Elp3 carrying the enzymatic function (Winkler et al., 2001).

1.2. The three roles of Elongator

The same team finally proposed that Elp3 only acetylates the histones H3 and H4 *in vivo* and that the importance of this acetylation is to maintain acetylation level of these histones. However, the acetylation can only occur *in vivo* if Elp3 is part of the holo-Elongator complex (Winkler et al., 2002). In 2006, acetylation of histone H3 by Elongator was shown in human cells. The same study also supports the role of Elongator in transcription regulation due to the decrease of genes expression following Elongator depletion (Figure 1.A) (Close et al., 2006).

The discovery by the Young lab that Elongator localisation is limited to cytoplasm questioned the transcriptional function and suggested additional roles (Pokholok et al., 2002; Rahl et al., 2005). In that context, it was proposed that Elongator regulates exocytosis through the control of the localisation of Sec2 (Figure 1.B). Indeed, this protein is a GEF (Guanine Exchange Factor) of Sec4, which binds the exocyst complex to regulate post-Golgi secretion vesicles. Elp1 possesses at its COOH terminus, a binding domain to Sec2 and regulates its localisation (Rahl et al., 2005). The link found between the human homologue of Elp1 (IKAP), with the neurodegenerative disease, Familial Dysautonomia (FD), led to the hypothesis that this disease is the consequence of the dysregulation of neuronal exocytosis (Rahl et al., 2005; Slaugenhaupt et al., 2001).

A third role of Elongator was suggested in the regulation of cell migration through tubulin acetylation (Figure 1.C). It was reported that mouse IKAP co-localizes with filamin A, an important actor of cytoskeleton migration. The depletion of IKAP in MEFs (Mouse Embryonic Fibroblast) leads to a mislocalization of filamin A and a migration defect, which can be rescued by the introduction of human IKAP. In contrast, the introduction of a version of IKAP harbouring the mutations leading to the FD disease failed to rescue the migration of MEFs (Johansen et al., 2007). Later, the Chariot team showed that Elongator depletion in human cells decreases the expression of genes implicated in cell mobility. The perturbation of mobility could be the cause of the FD neurological disease (Creppe et al., 2009). The resulting hypothesis is that the acetylation of tubulin is performed by the cytosolic form of Elongator in addition to the acetylation of histones, which is in accordance with the ability of acetylases to modify several substrates. The defects observed in FD disease patients may therefore result from both a tubulin acetylation and a histone acetylation defects, resulting in decreased cell mobility (Gardiner et al., 2007). This hypothesis was supported in 2009 by the Nguyen lab that reported that *Elp1/Elp3* deletions in mouse model decreased the level of tubulin acetylation leading to impairment of neuronal cell migration and branching (Creppe et al., 2009). At this time, a new question arose: is Elongator achieving all these different functions or is it responsible of one function that regulates the others? The answer to this question came with the discovery of yet another function of Elongator brought by studies of the *Kluyveromyces lactis*-secreted toxin, zymocin.

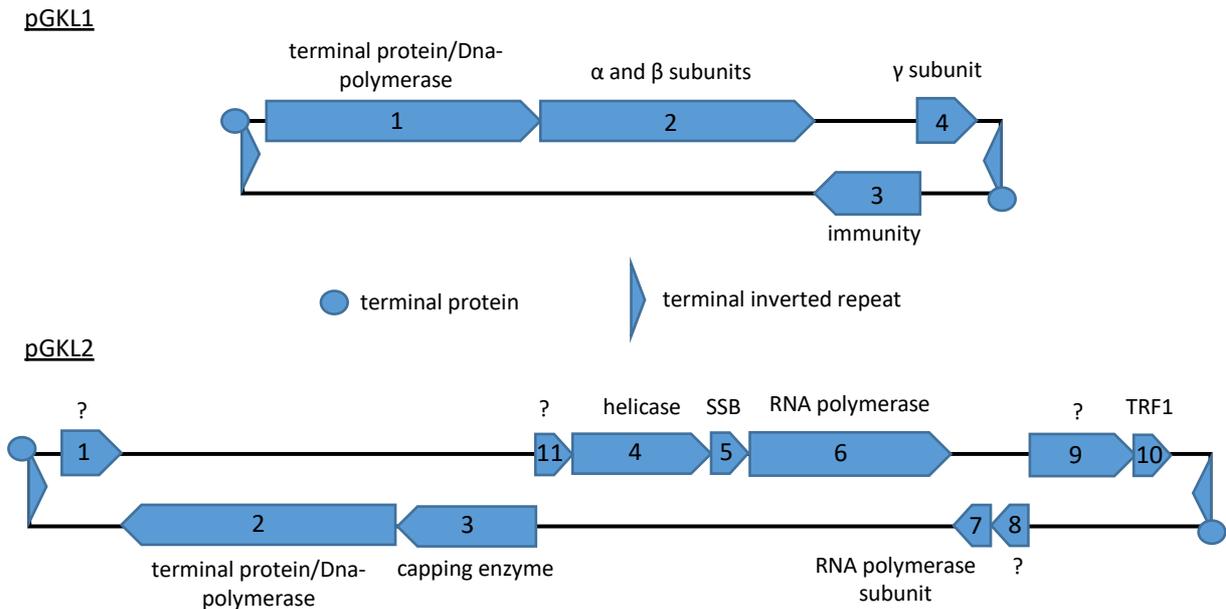


Figure 2. pGKL1 and pGKL2, linear plasmids encoding zymocin. Genetic organization of pGKL1 and pGKL2. ORF are indicated by number. Arrows indicate polarity of ORF and genes transcription. The gene functions are specified near the corresponding ORF. Question marks indicate the absence of information about the gene function. SSB: single strand binding protein, TRF1: terminal recognition factor 1. Adapted from (Schaffrath and Meinhardt, 2004; Vopálenský et al., 2019)

2. *Kluyveromyces lactis* and zymocin

Zymocin is a heterotrimeric, secreted toxin produced by some so-called “killer strains” of the yeast *Kluyveromyces lactis* to provide a growth advantage on fast-growing fermenting organisms such as *S. cerevisiae* where zymocin induces a lethal growth arrest in the G1 phase of cell cycle (Butler et al., 1991c). This toxin was the first eukaryotic ribonuclease discovered. It was found in 1981 when the Sakaguchi team was analysing linear plasmids (Gunge et al., 1981). They found two plasmids pGKL1 (8.8kb) and pGKL2 (13.4kb), which they described as “plasmid with killer character” (Figure 2). These two plasmids are cytoplasmic and have a high A/T bias (more than 70%) (Schaffrath and Meinhardt, 2004). Five years later, Boyd and Stark found that the plasmid pGKL1 encodes two mRNAs: the ORF2 encoding the two first subunits, α and β (~99 and 30kDa respectively), and ORF4 encoding the last subunit, γ (~28kDa). The pGKL2 plasmid encodes several maintenance mechanisms of pGKL1. It seems that the two first subunits (α and β) are translated as a single protein, which is cleaved by a Kex2-like protease, Kex1 (Schaffrath and Meinhardt, 2004; Stark and Boyd, 1986; Stark et al., 1990). The toxic activity of zymocin is carried by the γ -subunit also known as γ -toxin, which shows an ACNase activity (Anticodon nuclease) (Jablonowski and Schaffrath, 2007). The α -subunit possesses a chitin binding domain (CBD) required for binding to target cells and a chitinase motif to exert its native toxicity (Schaffrath and Meinhardt, 2004). It was shown that mutant yeasts lacking chitin are resistant to zymocin and that toxin treated with allosamidin, a specific inhibitor of insect, fungal and bacterial chitinases loses its toxic activity (Butler et al., 1991a). The α -subunit is also the only subunit with a N-linked oligosaccharide chain (Schaffrath and Meinhardt, 2004; Stark et al., 1990). The hydrophobic β -subunit is needed to let γ -subunit passes through the target cell membrane. Moreover, the protein sequence of this subunit has similarities with *E. coli* TolQ, a factor requires for colicins (*E. coli* endonuclease) to entry into the target cells (Schaffrath and Meinhardt, 2004; Stark et al., 1990). To prevent *K. lactis* to be impacted by its own toxin, the pGKL1 plasmid (ORF3) also encodes for an immunity protein (Schaffrath and Meinhardt, 2004). These three subunits form a discrete complex *in vivo* thanks to disulphide bonds which maintain holo-zymocin integrity. The β -subunit is bonded to γ -subunit and α -subunit possesses intramolecular disulphide bonds (Schaffrath and Meinhardt, 2004; Stark et al., 1990).

3. Zymocin brought a new function to Elongator

In order to identify the target of zymocin, several groups screened for mutants resistant to *S. cerevisiae* mutants. In 1991, Butler *et al.* found four mutants resistant to toxin called *ktil-4* (killer toxin insensitive). These mutants can be divided in two classes. The first class regroups genes that give resistance against exogenous zymocin (*ktil2* and *4*) and genes of the second class give resistance against the catalytic subunit of zymocin, even if the expression is intracellular (*ktil1* and *3*). They showed by that time that the intracellular expression of γ -toxin induces a reversible G1 arrest unlike the native toxin, which leads to cells death (Butler et al., 1991b). Currently, it is accepted that the intracellular expression of γ -toxin induced an irreversible G1 arrest and cell death. The same group continued their researches and discovered nine new *ktil* mutants; *ktil6-14*. They also figured out that the class I mutant *ktil2* is allelic to CAL1/CSD2, which encodes chitin synthase III. This finding was in agreement with the inability of native zymocin to cross cell membrane of class I mutants. A second part of the research was the discovery that overexpression of tRNA^{Glu}_{UU^C} gives zymocin resistance to sensitive budding yeast strains. These data supported the hypothesis that tRNAs may be the target of zymocin (Butler et al., 1994).

Two additional screenings were performed later to identify other genes providing sensitivity to zymocin. The first one was the screen of Kishida *et al.* in which eight different mutations

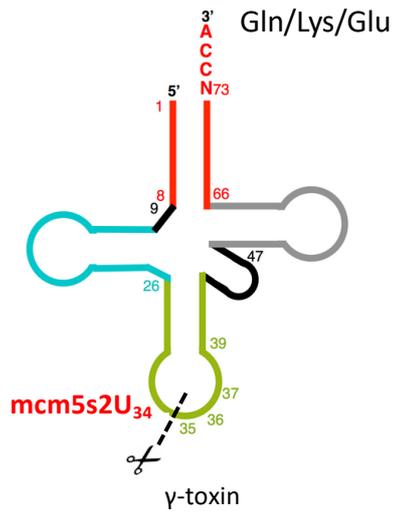


Figure 3. tRNA cleavage by γ -toxin subunit of zymocin. γ -toxin cleaves mcm5s2U modified tRNA^{Glu}_{UUC}, tRNA^{Lys}_{UUU} and tRNA^{Gln}_{UUG} between nucleotides 34 and 35 of the anticodon stem loop. Modified from (Tamura, 2015)

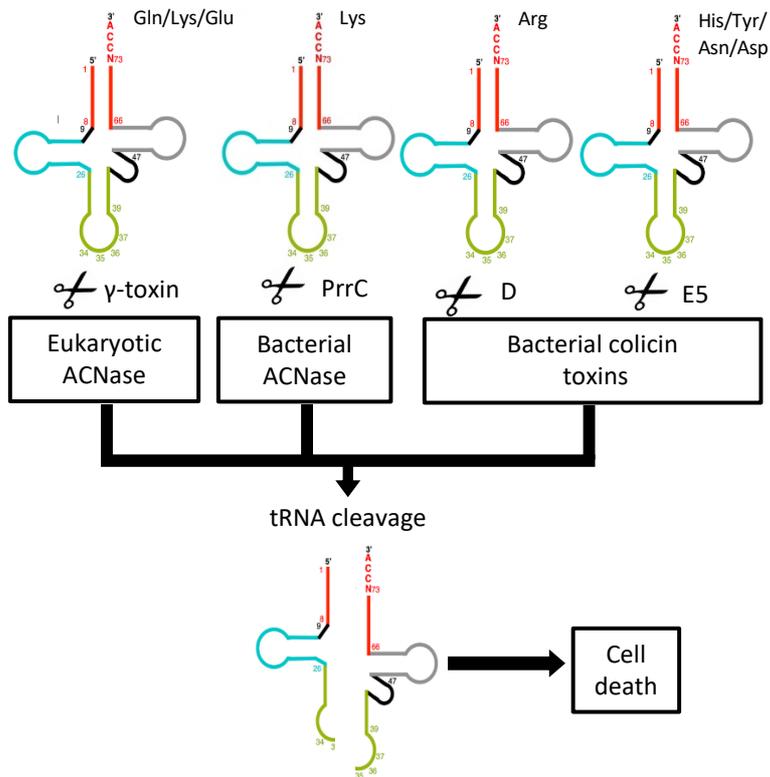


Figure 4. Target of microbial tRNases. tRNase secreted by microbes are used to face competition by targeting specific tRNAs. The consequence of this cleavage is the cell death. ACNase: AntiCodon Nuclease. Adapted from (Jablonowski and Schaffrath, 2007)

conferring resistance to γ -subunit of zymocin were found. They called them *iki1-5* (killer-insensitive). They argued that some of their mutants can be allelic in some of the *kti* mutants (Kishida et al., 1996). The last screen performed by Frohloff et al. identified five mutants resistant to γ -toxin, *tot1-5* (γ -toxin target). They also identified that *TOT1*, *TOT2*, *TOT3*, *TOT4* and *TOT5* were allelic to *ELP1/IKI3*, *ELP2*, *ELP3*, *KTI12* and *IKI3* respectively. The absence of any Elongator subunits resulted in normal growth in the presence of synthetic zymocin or *K. lactis*, which supported a close connection between zymocin and Elongator. At this time two hypothesis were proposed. The first one was that the target of zymocin was Elongator itself, which leads to a decreased transcription of genes required in G1 phase. The second one support the fact that the target of zymocin was a gene product downregulated in Elongator mutants (Frohloff et al., 2001). The link between Elongator and zymocin was enforced with the fact that *kti11* and *kti13* showed the same phenotypes as Elongator mutants and that Kti11 interacts with Tot3/Elp3 (Fichtner and Schaffrath, 2002).

The Byström lab clarified the issue when reporting in 2005, that the three 5-methoxycarbonylmethyl-2-thiouridine modified tRNAs ($\text{tRNA}_{\text{UUC}}^{\text{Glu}}$, $\text{tRNA}_{\text{UUU}}^{\text{Lys}}$ and $\text{tRNA}_{\text{UUG}}^{\text{Gln}}$) at nucleoside 34 ($\text{mcm}^5\text{s}^2\text{U}_{34}$) are the target of zymocin with the greatest affinity for $\text{tRNA}_{\text{mcm}^5\text{s}^2\text{UUC}}^{\text{Glu}}$ (Figure 3). The toxin is an endoribonuclease leading to tRNA cleavage into a 2', 3' cyclic phosphate and a 5' hydroxyl group. It was also shown that the toxin needs both the mcm^5 and s^2 moieties of the modification to efficiently cut tRNA anticodon stem loop (Lu et al., 2005). This mode of action is not unique, as many bacteria use a similar strategy as D or E5 colicins, which cleave modified tRNA^{Arg} and $\text{tRNA}^{\text{His,Asp Tyr,Asn}}$ respectively or Prrc ACNase, which cleaves modified tRNA^{Lys} (Figure 4) (Jablonowski and Schaffrath, 2007). Finally, the Byström laboratory and the Shuman laboratory reported the importance for zymocin cleavage of the anticodon stem loop conformation and the composition of the anticodon stem loop of $\text{tRNA}_{\text{UUC}}^{\text{Glu}}$ ($\text{U}_{33}\text{U}_{34}\text{U}_{35}\text{C}_{36}\text{A}_{37}$), which could explain the tRNA cleavage difference of efficiency between individual mcm^5s^2 -modified tRNAs (Jain et al., 2011; Lu et al., 2008).

4. Current knowledge about Elongator

4.1. A single function for Elongator

The Byström lab discovered that some tRNA species show decreased level of mcm^5 and ncm^5 (5-carbamoylmethyl) modifications on uridine 34 (wobble position) in *elp1-6* or *kti11-13* mutants. The *elp1-6* and *kti11* and *12* mutants abolish the synthesis of the two modifications but *kti13* only decreases the level of modified nucleosides. This is probably explained by a regulatory role of Kti13. However, the thiol group of $\text{tRNA}_{\text{UUC}}^{\text{Glu}}$ is still detectable in mutants meaning that thiolation function is carried by other actors (Huang et al., 2005).

The localization of the complex is unclear and leads to misunderstanding of Elongator role. In accordance with its function of tRNA modification, the bulk of Elongator is found in the cytoplasm. However, when first discovered, Elongator was thought to be nuclear. Other studies showed that small amount of Elongator also precipitated with unspliced RNAs found in nucleus arguing for nuclear localization (Gilbert et al., 2004; Petrakis et al., 2004).

From these works, the idea emerged that Elongator may only targets tRNA modifications that would in turn affect a variety of other cellular processes. This idea was supported by the already known translational defect in other organisms as *Shigella flexneri* and *Salmonella enterica* due to the lack of nucleoside modifications at anticodon. The *Shigella flexneri* example showed that a translational defect could lead to a transcriptional defect. Based on these critical genetic data, the Byström lab proposed the hypothesis that the tRNA modification is the principal, if not

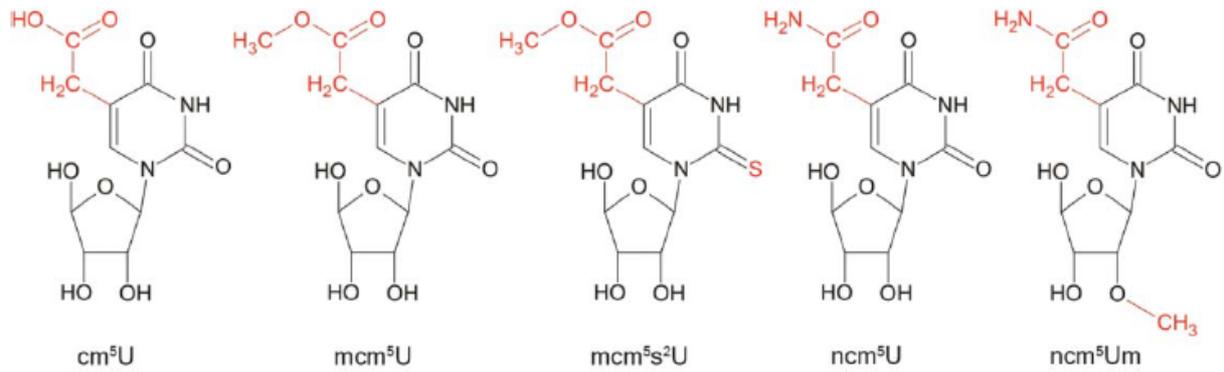


Figure 5. Structure of wobble uridine modifications. cm^5U : 5-carboxymethyluridine; mcm^5U : 5-methoxycarbonylmethyluridine; $\text{mcm}^5\text{s}^2\text{U}$: 5-methoxycarbonylmethyl-2-thiouridine; ncm^5U : 5-carbamoylmethyluridine; ncm^5Um : 5-carbamoylmethyl-2'-O-methyluridine. The modifications are highlighted in red. cm^5U is an intermediate of other modifications. From (Karlsborn et al., 2014)

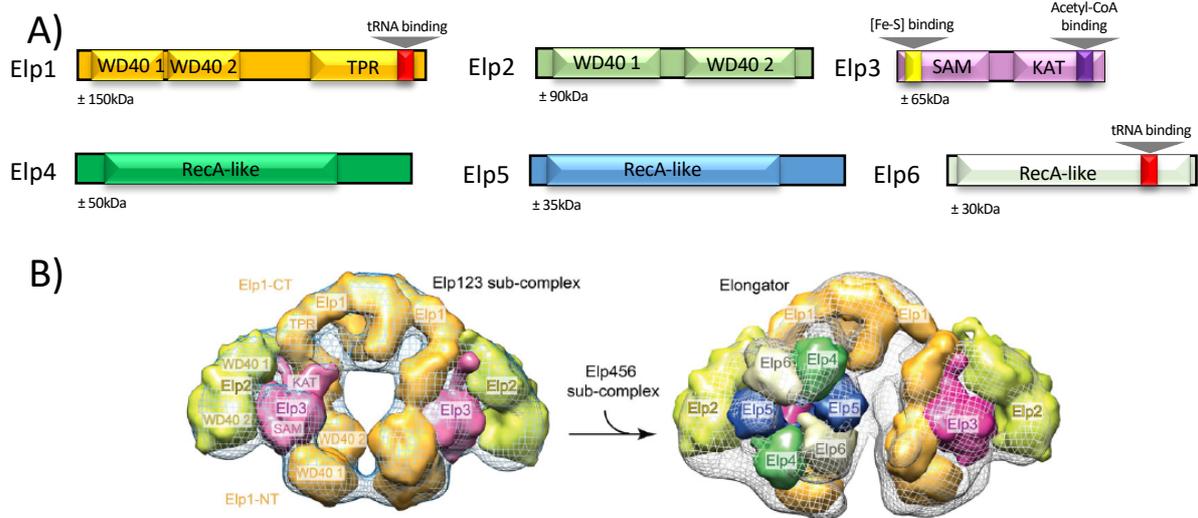


Figure 6. Elongator complex and Elp1-6 subunits. A) Schematic representation of structure and domain composition of Elongator subunits. B) Conformational structure of Elp123 subcomplex and Elongator. The colour code of subunits is the same for A. and B. WD40: WD40 β -propeller domain, TPR: tetratricopeptide repeat domain, SAM: radical S-adenosyl methionine binding domain, KAT: Lysine AcetylTransferase domain, NT: N-terminal, CT: C-terminal. Adapted from (Dalwadi and Yip, 2018; Dauden et al., 2018; Glatt and Müller, 2013)

only, function of Elongator and that all the observed phenotypes of the mutants result from translational defects (Huang et al., 2005). A decisive evidence supporting this possibility was that the decreased level of H3 acetylation and the mislocalization of Sec2, two previously reported phenotypes resulting from the absence of Elongator, could both be suppressed by the overexpression of the hypo-modified tRNA_{s2UUU}^{Lys} and of tRNA_{s2UUG}^{Gln}. These data supported that defects in tRNA modification were causal to the reported phenotypes of Elongator mutants (Esberg et al., 2006).

4.2. Structure of Elongator

Nowadays, it is known that Elongator is composed of six subunits each of them in two copies. These subunits form two sub-complexes, which once assembled, are responsible for the synthesis of the cm⁵ moiety of several modifications (ncm⁵U, mcm⁵U, mcm⁵s²U and ncm⁵Um) of some tRNAs on their wobble uridine (Figure 5). The deletion of any subunit inactivates Elongator. Elp1 (~150kDa) possesses two WD40 β-propeller domains at N-terminal and a TPR (Tetratricopeptide repeat) domain at C-terminal (Figure 6). Its name, IKK-complex-associated protein, came from the thought it was a scaffold protein of the IκB kinase complex in human cells (Cohen et al., 1998). It is known that it acts as a scaffold protein for the entire complex and shares high affinity for Elp2 and Elp3 unlike Elp456 that dissociates in the presence of high salt concentration. Two regulatory factors (a kinase, Kti14 and a phosphatase, Sit4) are known to balance the phosphorylation of Elp1 at the C-terminal end, which plays a role in Elongator function (Dalwadi and Yip, 2018; Glatt and Müller, 2013; Kolaj-Robin and Séraphin, 2017). The second largest subunit, Elp2 (~90kDa), contains also two WD40 domains but its function is less clear. It seems to participate in the scaffold function. Elp3 (~65kDa) is the catalytic subunit of Elongator and harbours two catalytic domains, rSAM and KAT, which bind SAM and Acetyl-CoA respectively. The remaining subunits, Elp456, form a RecA-like ATPase ring composed of 2 copies of each Elp456 protein. It seems to have an ATP-dependent affinity for the anticodon stem loop of tRNAs and so could play a role in the release of modified tRNAs (Dalwadi and Yip, 2018; Glatt and Müller, 2013; Kolaj-Robin and Séraphin, 2017).

4.3. The roles of mcm5s2 modification

The 5-methoxycarbonylmethyl-2-thiouridine is a tRNA modification of the wobble uridine (at position 34). This complex modification requires a set of genes identified by a genetic screen in *S. cerevisiae* (Huang et al., 2008). The whole synthesis is a three-step process (Figure 7). The first one is the cm addition at carbon 5 of the uridine 34 (by Elp1-6) followed the methylation of cm⁵ (by Trm9 and Trm112) resulting in mcm⁵. Finally, the carbon 2 of the uridine is thiolated by a relatively well-known pathway composed of 11 proteins. However, the order of these three steps is still debated (Dalwadi and Yip, 2018; Dewez et al., 2008; Kolaj-Robin and Séraphin, 2017; Leidel et al., 2009). This modification has three different roles to the cell: (1) the coordinated, codon-specific control of translation, (2) the reprogramming of translation and (3) the maintenance of proteome integrity by counteracting protein aggregation.

4.3.1. Coordinated, codon-specific control of translation

The role of mcm⁵s² modification is to regulate the translation through the stabilization of some codon – anticodon interactions (Figure 8.A) (Murphy et al., 2004; Yarian et al., 2000). mcm⁵s² tRNA_{UUC}^{Glu}, tRNA_{UUU}^{Lys} and tRNA_{UUG}^{Gln} modifications enhance the recognition of A and G ending codons and so the translation rate (Johansson et al., 2008). Interestingly, the use of modified tRNAs coupled with a skewed codon bias can also participate in a coordinated regulation of translation of groups of mRNAs enriched for codons recognized by the modified tRNAs and therefore under the dependency of Elongator (Bauer et al., 2012).

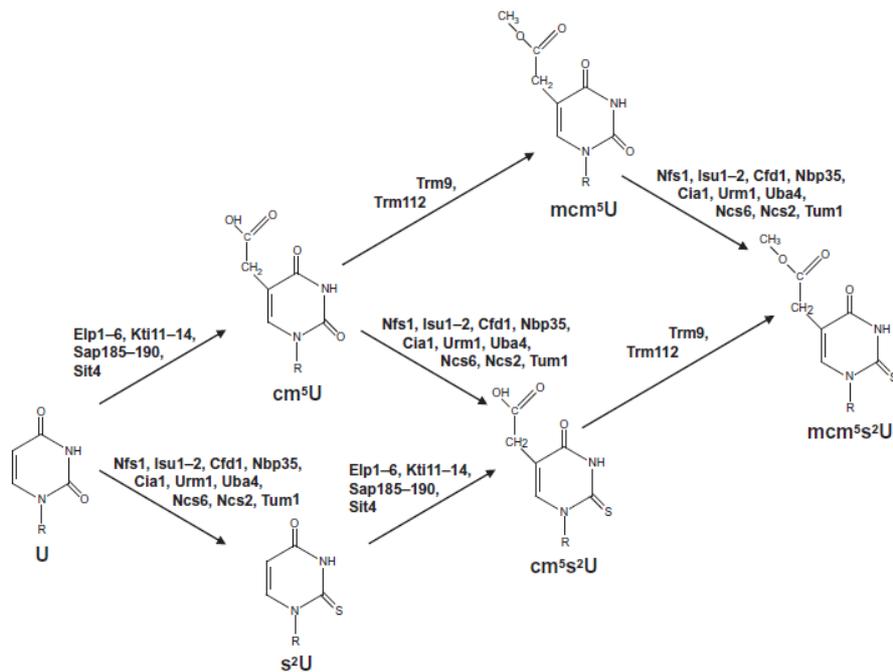


Figure 7. mcm⁵s²U modification pathway. Steps in the formation of the double mcm⁵s²U₃₄ modification are presented with the intermediate forms of U₃₄. The order of these steps is still mostly speculative. From (Kolaj-Robin and Séraphin, 2017)

4.3.2. Ability of reprogramming translation

In addition to this control of translation, Elongator is able to regulate the translation of genes depending on the conditions (Figure 8.B). It is already known that the phosphorylation of Epl1 is regulated by the Kti14 kinase and the Sit4 phosphatase. Moreover, another study showed in *S. pombe* that the cell fate between growth or differentiation, controlled by the balance between the TORC1 and TORC2 complexes, is regulated by Elongator. Elongator induces differentiation by sustaining the translation of subunits of TORC2 complex while inhibiting TORC1 through the increased translation of TORC1 inhibitors. The regulation relies on the skewed codon content of some mRNAs that are enriched for codons read by the modified tRNAs (Candiracci et al., 2019; Hermand, 2020).

4.3.3. Maintenance of proteome integrity by counteracting protein aggregation

The mechanism for maintenance of proteome integrity by Elongator is linked to the speed of translation that depends on the codons-anticodons recognition (Figure 8.C). It was proved by Nedialkova D. and Leidel A. using ribosome profiling that hypo-modified tRNA^{Glu}_{UUC} and tRNA^{Lys}_{UUU} slowed the translation rate through decreased speed for recognition of their cognate codons in *S. cerevisiae* and *C. elegans*. The disruption of translation rate has for consequence the misfolding and aggregation of proteins giving rise to perturbation of proteome integrity (Nedialkova et al., 2015). The protein aggregation due to the lack of mcm⁵s² modification was also observed in mice and human. This phenomenon could be the basis of neurodegenerative and neurodevelopmental diseases development associated to Elongator (described below) as protein aggregation is an important hallmark of these diseases (Hawer et al., 2019).

5. Importance of Elongator

5.1. Conservation of Elongator

This complex mainly studied in yeast is well conserved in all eukaryote organisms. Elongator is found in plants (*Arabidopsis thaliana*), in nematodes (*Caenorhabditis elegans*), in fruit flies (*Drosophila melanogaster*), in mouse (*Mus musculus*), in humans and also in archaea (*Methanocaldococcus infernus*) (Hermand, 2020; Karlsborn et al., 2014). The absence of Elongator and mcm⁵s² modification in bacteria is explained by the presence of a different pathway that leads to the modification of the uridine of the corresponding tRNAs with the related 5-methylaminomethyl-2-thio modification (mnm⁵s²). The role of this modification is similar to Elongator modification in yeast (Ikeuchi et al., 2006).

The importance of this complex is shown by the effects of its deletion on several organisms. Indeed, the inactivation of Elongator, while viable in yeast, strongly affects the response to various environmental conditions due to the lack of tRNA modifications. The *ELP1* mutation in the yeast *S. cerevisiae* results in decreased growth rate, temperature sensitivity and resistance to the *Kluyveromyces lactis* killer toxin (Frohloff et al., 2001; Otero et al., 1999). In a mouse model, the absence of this modification due to deletion of *Epl1* (*IKBKAP*^{-/-} mouse) is lethal and shows many embryogenesis defects including brain formation and vascularisation defects (Chen et al., 2009b). Defects in the development of neurons were also reported in *Caenorhabditis elegans* following the inactivation of Elongator. It was shown that these defects result from the decreased translation rate of neuropeptides and neurotransmitters (Chen et al., 2009a). In *Drosophila*, the deletion of *ELP3* gene induces a pupal deletion preceded by growth disturbances (Walker et al., 2011). In *Arabidopsis thaliana*, cell proliferation defects had been observed after the deletion of *ELP1*, *ELP3*, *ELP4*, or *KTI12* gene homologues (Karlsborn et al., 2014). These few examples support the high conservation of Elongator function and roles in Eukaryotes (Karlsborn et al., 2014; Li et al., 2005; Mehlgarten et al., 2010; Selvadurai et al., 2014).

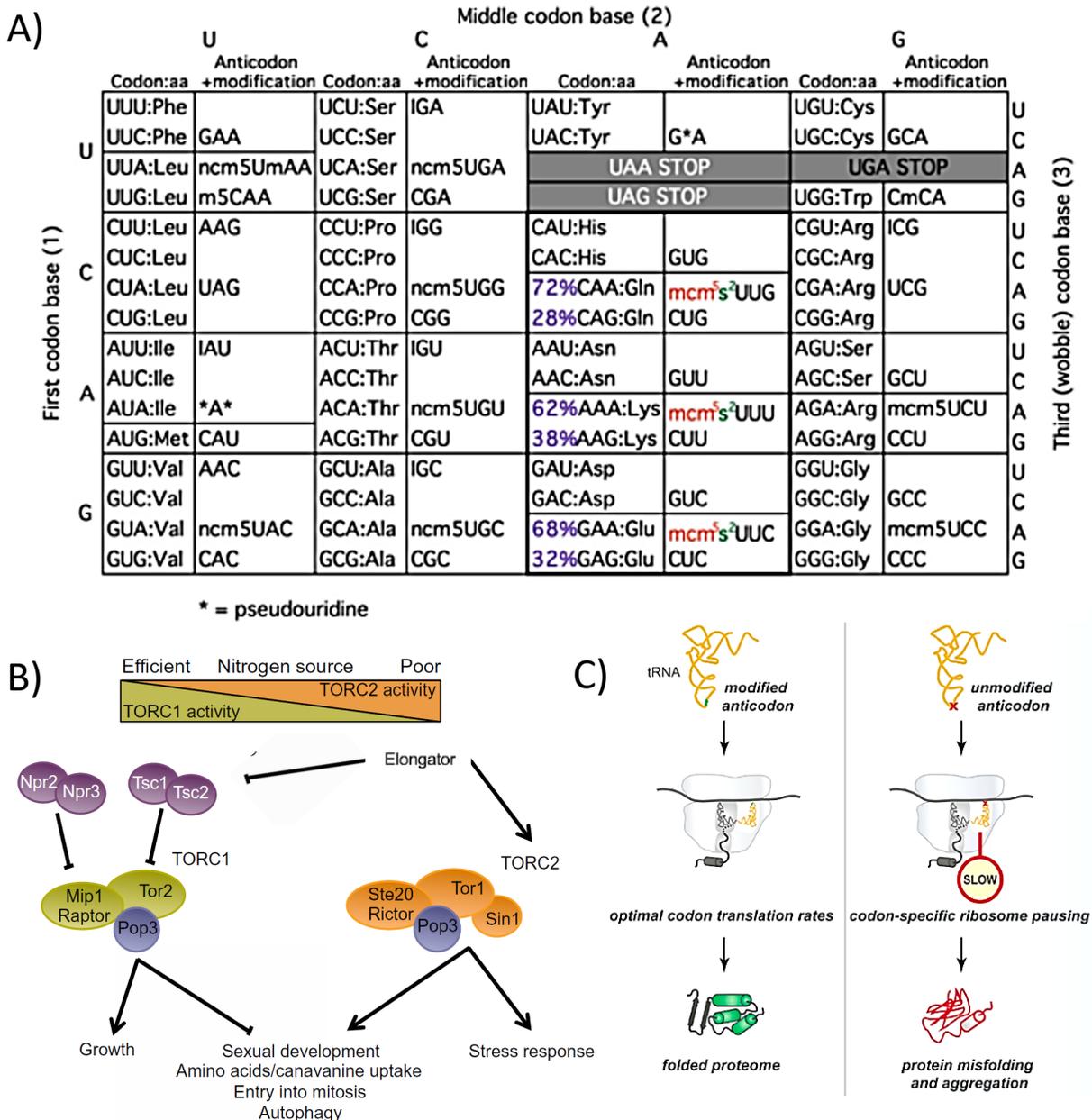


Figure 8. Roles of mcm⁵s² modification. A) The coordinated, codon-specific control of translation: The genetic code with Elongator-dependent anticodon modification shows codon bias for mcm⁵s²U modified tRNA^{Glu}_{UUC}, tRNA^{Lys}_{UUU} and tRNA^{Gln}_{UUG}. From (Hermand, 2020). B) Ability of reprogramming translation: Example of the translation reprogramming in starvation conditions, Elongator reprograms the cell fate via regulation of TORC2. The TORC1 inhibition is made by the increased translation of TORC1 inhibitors. Adapted from (Candiracci et al., 2019) C) Maintenance of proteome integrity: The absence of modification on uridine 34 slow down the translation inducing a misfolding and aggregation of proteins. From (Nedialkova et al., 2015).

5.2. Diseases linked with Elongator

The importance of Elongator, due to its broad range of action, makes it a central actor of multiple pathologies. As well as many tRNA modification genes, implicated in several diseases, Elongator is not an exception (Hawer et al., 2019). Actually, this complex is associated with several diseases. Even if the majority of them are related to brain functions, some of them are cancers of any kind presenting Elongator overexpression (Figure 9).

5.2.1. Neurodegenerative and neurodevelopmental diseases

Few years after the discovery of Elongator, a point mutation in the exon 20 donor splice site of *ELP1* was found to be the cause of Familial Dysautonomia, an autosomal recessive disease, which impacts the development and survival of sensory, sympathetic and some parasympathetic neurons (Slaugenhaupt et al., 2001). This mutation leads to exon 20 skipping and to a truncated form of ELP1 (~79kDa) preventing the formation of Elongator. Another mutation, less common causes a missense mutation in exon 19 which disrupts a phosphorylation site (Anderson et al., 2001; Slaugenhaupt et al., 2001). Others studies linked ELP3 with amyotrophic lateral sclerosis (ALS), a fatal neurodegenerative disorder. Its progression leads to muscles weakness, atrophy and spasticity due to neuron degeneration. The first study demonstrated, thanks to three different organisms, human, *D. melanogaster* and *C. elegans*, that loss of function mutations in Elp3 lead to the degeneration of neurons and the disruption of axonal biology. Moreover, this study performed an association study of microsatellite markers which found three alleles of Elp3 linked with ALS. The presence of one of these risk-alleles of *ELP3* correlates with a lower level of ELP3 expression in the human brain (Simpson et al., 2009). In the second study, the presence of Elp3 shows a benefit effect on disease progression in ALS mouse model or in ALS zebrafish model. Indeed, the insoluble level of mutant SOD1, a protein responsible for some case of familial ALS, increased following the disruption of Elp3 (Bento-Abreu et al., 2018). Older studies also reported cases of diseases with some Elongator subunits. It is the case of microdeletions of *ELP4* which are related to language impairment, autism spectrum disorder and mental retardation (Addis et al., 2015). Earlier, the same team had shown that variants of ELP4 are linked with Rolandic Epilepsy, the first disease related with ELP4 (Strug et al., 2009). ELP2 was also related to neurodevelopmental defect, the Intellectual Disability. A study had shown that two brothers with Intellectual Disability were carriers of two variant alleles of ELP2, p. H271R and p. R57W, which present a substitution of a positive charged, basic amino acid with another one positive, basic amino acid and with a non-polar, neutral amino acid respectively (Cohen et al., 2015). A last example of disease associated to Elongator subunit mutation is the mouse ataxia-like phenotype resulting to point mutation in *Elp6* encoding gene. This phenotype is supported by Purkinje neurons degeneration (Kojic et al., 2018).

5.2.2. Cancers development

Elongator is not only associated to neurodegenerative and neurodevelopmental diseases. Other studies have linked Elongator with several cancers. The team of Chariot discovered that Elp5 and Elp6 are important for tumour initiation and cell migration in melanoma. They reported that the depletion of Elp5 and Elp6 impacts the motility of melanoma-derived cells at the same level as Elp1 and Elp3 depletion (Close et al., 2012). They had also shown that Elp3 and Ctu1 (Ncs6 human counterpart), the enzyme implicated in U₃₄ thiolation, promote the metastasis of breast cancer due to mcm⁵s²U₃₄ modification (Figure 10). The mechanism passes through the expression of the Dek protein encoded by an mRNA with a skewed codon content for AAA, GAA, and CAA. Dek is a pro-oncogenic protein implicated in IRES-dependent translation of Lef-1. This last one is needed to ensure cancer cell motility and cell migration. As a result of the codon content of its mRNA, Dek is dependent on Elp3, Ctu1 and the corresponding modifications for efficient translation. In addition, these enzymes were shown to be overexpressed in breast cancer (Delaunay et al., 2016). Previously, the same team showed that

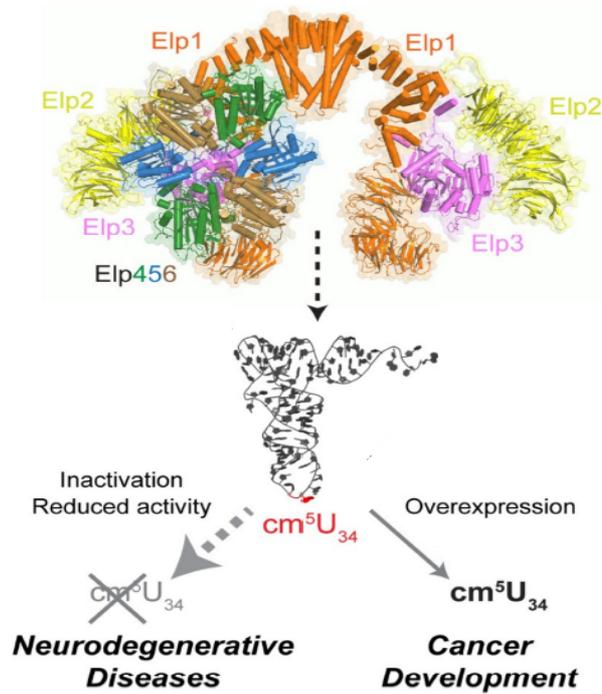


Figure 9. Elongator linked with diseases. Elongator was linked to several diseases which can be classed in two groups: Neurodegenerative diseases characterized by the absence of modification due to mutation, and cancer development characterized by the overexpression of the modification. The top of the figure represent the fully assembled Elongator complex with the 6 subunits: Elp1 in orange, Elp2 in yellow, Elp3 in pink, Elp4 in green, Elp5 in blue and Elp6 in brown. Adapted from (Hawer et al., 2019).

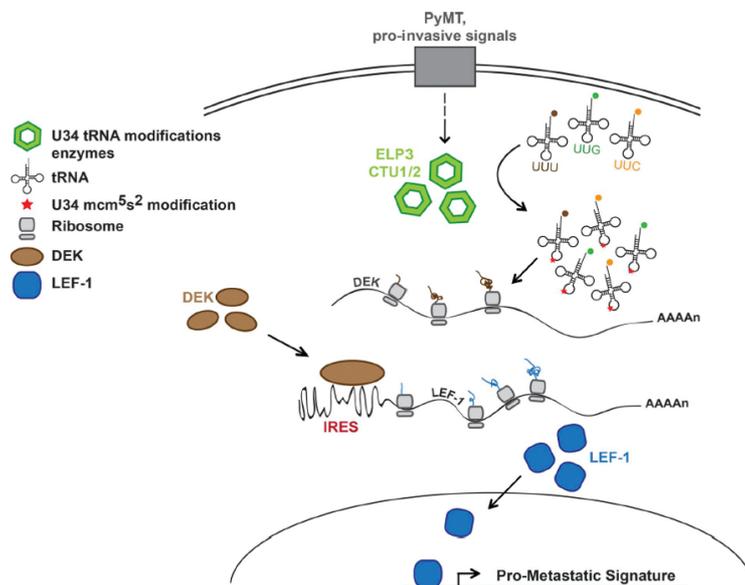


Figure 10. Model for implication of mcm5s2uridine modification in breast cancer metastasis. Pro-invasive signals increase tRNA modifications through Elp3 and Ctu1/2 activation. The increased level of modified tRNAs allows high level of Dek translation, which in turn increases Lef-1 translation level. The Lef-1 protein is required for pro-metastatic signature genes induction. From (Delaunay et al., 2016)

Elp3 plays a role in the maintenance of a specific pool of Lgr5⁺/Dclk1⁺/Sox9⁺ stem cells. These cells lead to the initiation of Wnt-driven intestinal tumours. Similarly to the previous cases, an enrichment for the AAA codon in the Sox9 mRNA makes it dependent on Elongator for high-level expression (Ladang et al., 2015).

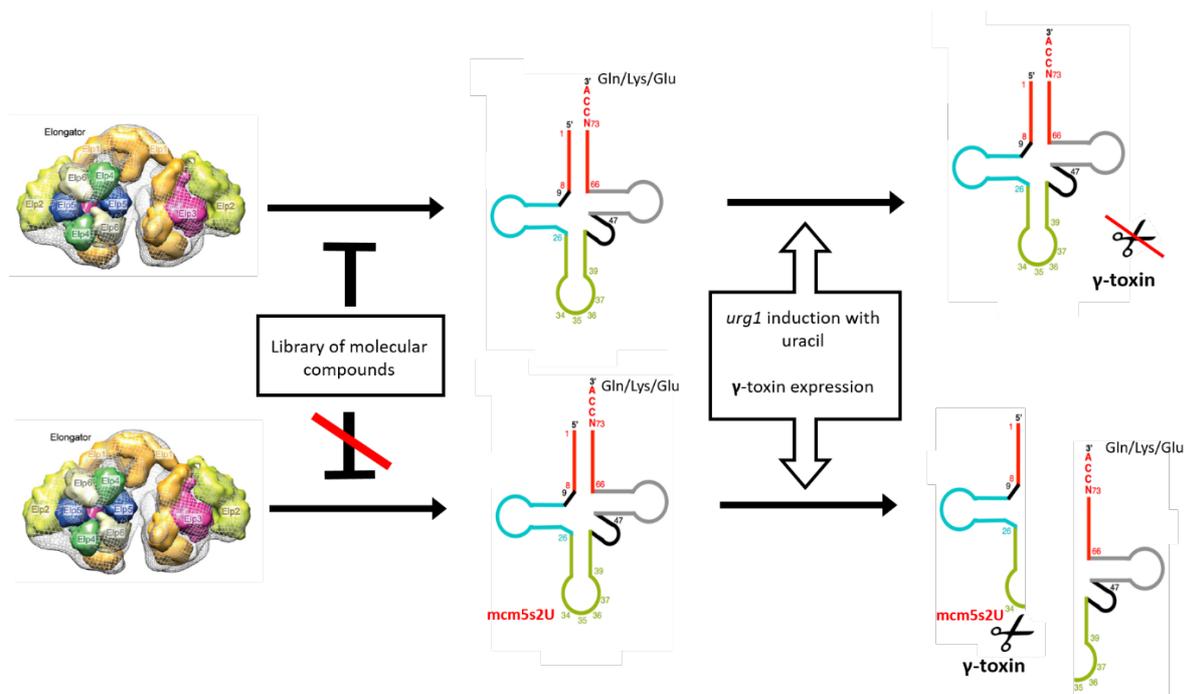


Figure 11. Detection system of mcm5s2uridine modification synthesis inhibition. The read-out relies on the ability of γ -toxin to cleave mcm⁵s²U modified tRNAs. If a compound inhibits Elongator, the unmodified tRNA is not a target of γ -toxin (expressed under the control of the *urg1* promoter). Conversely, if the compound does not affect Elongator, the modified tRNA is targeted by γ -toxin, resulting in cell death. Modified from (Dauden et al., 2018; Tamura, 2015).

Presentation of the project

1. Goal of the research

In this project, we plan to use yeast to screen a library of chemical compounds in order to select Elongator inhibitors (Figure 11). The screening requires several preliminary sets up to make it feasible and efficient. Indeed, even if Elongator is relatively well conserved among eukaryotes, it remains that approximately 750 million years of evolution separate yeasts and humans. Consequently, an important part of the work will be dedicated to the generation of a humanised *S. pombe* strain where all the subunits of the Elongator complex correspond to the human proteins. Moreover, a large-scale screen requires to construct an efficient read-out system to determine if a chemical compound has an inhibitory effect. To this end, we are going to use zymocin, which is a toxin naturally expressed by the budding yeast *Kluyveromyces lactis*. The particularity of this toxin is to specifically kill the organisms expressing Elongator because it targets the anticodon stem loop of the mcm⁵s² modified tRNAs, where it introduces a single strand break. The purpose of my project is to generate transgenic yeast expressing the gene encoding the toxic subunit of zymocin under the regulation of an inducible promoter to allow expression only after incubation with the chemical compound.

2. The model organism

We decided to use the fission yeast as model organism due to the powerful genetic tools available for this organism. This eukaryote cell is the smallest eukaryote fully sequenced. Its 13.8Mb haploid genome is divided in three chromosomes of 5.7, 4.6 and 3.5Mb presenting large heterochromatin centromeres (Forsburg, 2003). The advantages of the yeast compared to human cells are the facility of utilisation, the time saving of culture and results obtaining and the simple genetic manipulations while still being relatively relevant as a model system for studies mammal cells. The fission yeast is by several aspects closer to human cells than *S. cerevisiae*. For example, more genes of *S. pombe* have human homologues compared to budding yeast. Moreover, the centromeres of our model organism are relatively close to mammal centromeres. It is also the case of division realised by fission rather than by budding (Forsburg, 2003). Two last examples are the distance between the TATA box and the transcription start point (*tsp*) of 25 to 40bp and the more conserved consensus of intron splice site between mammalian cells and *S. pombe* (Maundrell, 1990) resulting in efficient splicing of human introns by *S. pombe* but not *S. cerevisiae*.

3. The choice of inducible promoter

The fission yeast gives us several possibilities of inducible promoters to express our toxin. The two most widely used are *Pnmt1* and *Purg1*. The gene of *nmt1* (no message in thiamine) was the first fully regulatable gene in *S. pombe*. It was discovered in 1990 by Maundrell (Maundrell, 1990). Later, with its team, Maundrell et al. showed that the TATA box of *Pnmt1* (5'-ATATATAAA) located 25bp of the *tsp* can be mutated to decrease the level of expression when repressed or induced. They generated two new inducible promoters named *Pnmt41* and *Pnmt81* by deletion within the TATA box (5'-ATAAA and 5'-AT respectively). According to them, the expression, when induced, is approximately 7 and 80 times weaker respectively compared to the full-length *nmt1* and, when repressed, is 16 and 250 times weaker respectively than *nmt1* (Basi et al., 1993). The main problem is the time of induction of these promoters. It needs between 15 and 21 hours after removing of thiamine to reach full induction (Watson et al., 2013). The second promoter is *Purg1*, which is the promoter of the gene encoding for Urg1, Uracil-regulated protein 1. This promoter, as its name suggests, is regulated by the presence of uracil. The deletion of this gene does not impact the physiology of the fission yeast, which makes it an interesting inducible promoter. Moreover, a powerful advantage is the short

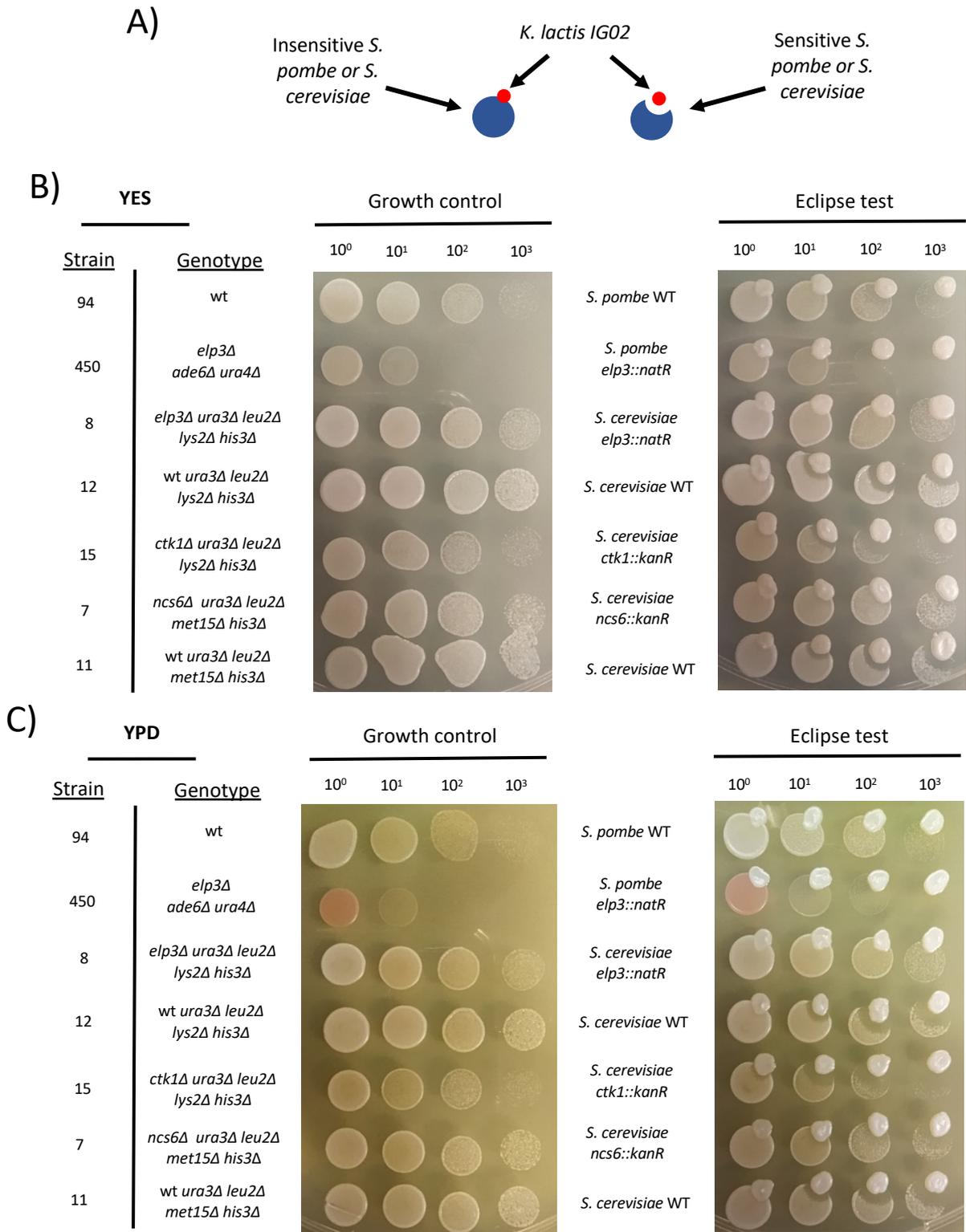


Figure 12. Assessment of extracellular zymocin on *S. pombe*. A) Eclipse test consists in a growth competition between drops of yeasts and a small patch of *K. lactis* IG02 strain. The apparition of a no growth ring means that the yeast tested is sensitive to secreted zymocin. B) Eclipse test on YES (growth media for *S. pombe*). The yeasts have grown 1 day at 32°C. C) Eclipse test on YPD (growth media for *S. cerevisiae*). The yeasts have grown 1 day at 32°C.

induction time, which is reported to be approximatively of 30 minutes after uracil addition. Another advantage is that uracil addition does not disturb the expression of most other genes. However, the default of this promoter is the necessity to integrate the gene of interest at the locus of *urg1*, which often results in a significant expression leakage (Watson et al., 2013; Watt et al., 2008). In view of the induction time and the advantage of adding uracil rather than remove thiamine, which is harder for a screening, we have decided to try to integrate the gene of γ -toxin under the regulation of *Purg1*.

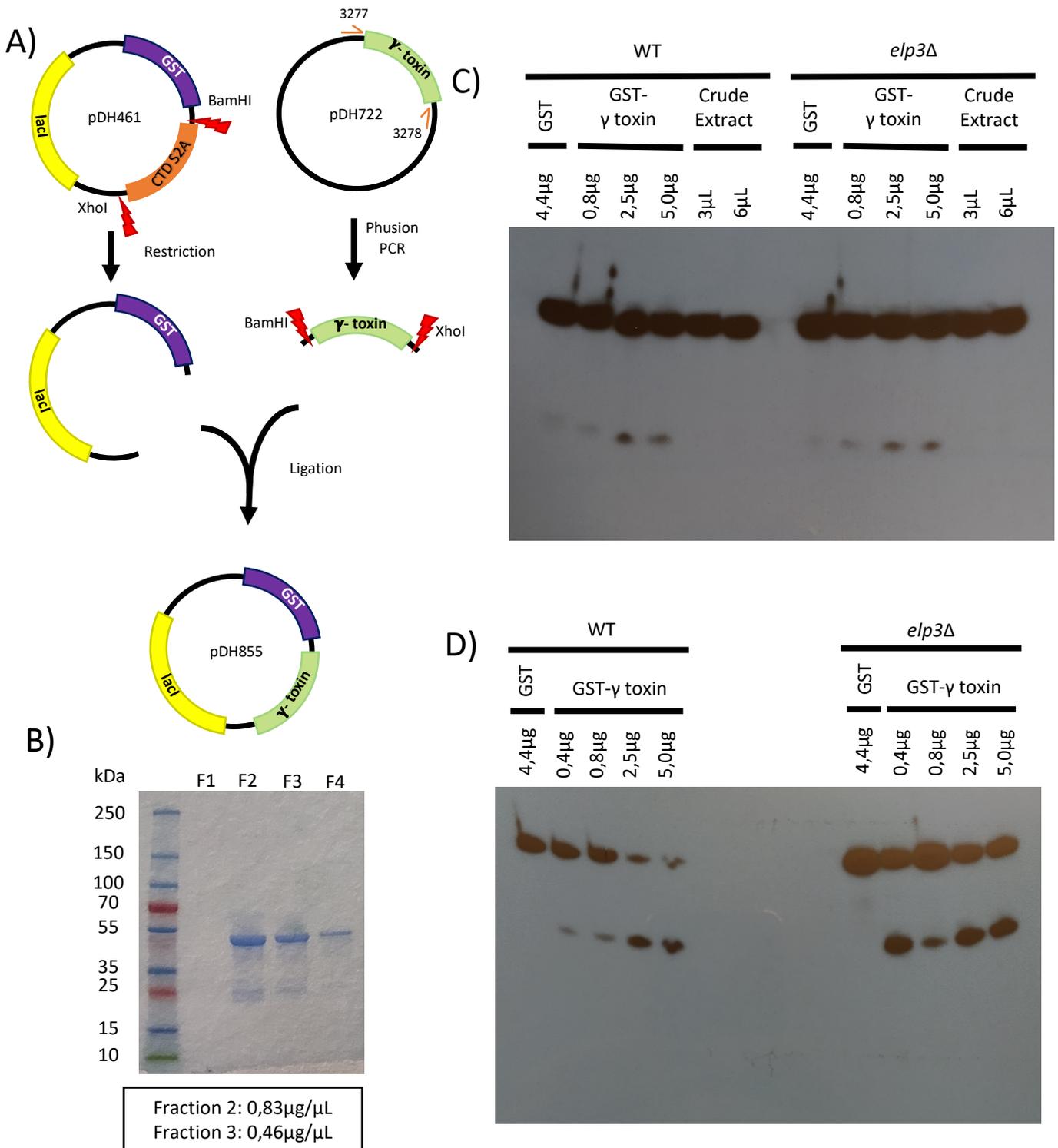


Figure 13. Cleavage of tRNAs by GST- γ -toxin. A) A GST-tagged γ -toxin gene was constructed with Phusion PCR by γ -toxin amplification from pDH722 with primers 3277 and 3278. The restriction had been realised with BamHI/XhoI restriction enzymes and the ligation into pDH461 (pGEX-4T1). B) Protein migration of the four fractions of GST- γ -toxin purified on columns. Molecular weight of GST- γ -toxin: 53kDa. Gel was coloured with Coomassie blue before drying. The quantification of concentration was realised by Bradford assay. C) Northern blot realised on sRNA enriched samples from strain 2060 (WT) and strain 2061 (*elp3 Δ*) after 1 hour of GST, GST- γ -toxin or crude extract cleavage. The membrane was exposed 6 hours. Probes used: 5'tRNA^{Lys} (3402) D) Northern blot realised on sRNA enriched samples from strain 2060 (WT) and strain 2061 (*elp3 Δ*) after 1 hour of GST or GST- γ -toxin cleavage. The membrane was exposed 5 hours. Probes used: 5'tRNA^{Glu} (3438) for WT and 3'tRNA^{Glu} (3439) for *elp3 Δ* .

Results and discussion

In order to perform a screening for compounds inhibiting the Elongator complex, we need an efficient read-out system to select these potential inhibitors. To this end, we choose to express the γ -subunit of zymocin that specifically requires the activity of Elongator to cleave its target tRNAs. Our first goal was therefore to construct a *S. pombe* strain expressing the γ -toxin. However, a second constraint is the necessity to control the expression of this toxin with a regulatable promoter. In *S. cerevisiae* the best-known inducible promoter is pGAL, which is strongly and quickly activated by the addition of galactose. *S. pombe* has no equivalent but the promoter of the *urg1* gene was reported to be strongly induced by the addition of Uracil and was chosen to induce the expression of γ -toxin. We first tested the sensitivity of fission yeast to the toxin.

1. Assessment of γ -toxin action on fission yeast

The zymocin secreted by *K. lactis* is a toxin efficiently killing *S. cerevisiae*. The gene encoding its catalytic subunit, called γ -subunit or γ -toxin, was cloned and shown to encode an endonuclease targeting the anticodon of three tRNAs bearing the mcm⁵s² modification. However, the possibility to cleave tRNAs from other organisms albeit likely based on the remarkable conservation of tRNA sequence and structure is yet to be proved. As the use of the toxin is the base of the screening strategy, it was important to first test it on the fission yeast.

1.1. Extracellular effect of γ -toxin

Among the differences resulting from the broad evolutionary distance between budding and fission yeasts, there is the composition of the cell wall. Considering that the α -subunit presents both a chitin binding domain and an exochitinase activity, it was interesting to test if extracellular zymocin has an effect on *S. pombe*. The simplest way to test the effect of the toxin on *S. pombe* is the eclipse assay (Figure 12). The principle is to deposit drops of the yeast strains to be tested at several dilutions and to overlay a small patch of *K. lactis*. If a no-growth ring appears around the *K. lactis* patch, it means that the tested strain is sensitive while confluent growth of the two species indicate resistance to extracellular zymocin. We have tested two strains of *S. pombe*, a wild type and an *elp3* deleted strain and 5 strains of *S. cerevisiae*, two wild type, an *elp3* deleted, a *ncs6* deleted, which lacks the gene encoding for the enzyme responsible of the thiolation of the uridine 34 and is also expected to be resistant. The unrelated *ctk1* deletion was used to exclude an effect of the *kanR* resistance selection marker. In addition, we also tested an effect of the rich medium used: YES, that is the used within the fission yeast community and YPD used by budding yeast researchers. The main difference is the presence of peptone in YPD and the fact that the YES is supplemented with several amino acids and bases.

The first things that we observed is the decreased growth rate of the Elongator mutant strains in both species, as previously reported. The cell density also impacted the killing ability of *K. lactis*. An mcm⁵s² modification-dependent eclipse was clearly visible for the *S. cerevisiae* wild type strain, as reported. When fission yeast was tested, these effects were much weaker and *K. lactis* appeared to be very inefficient at competing with *S. pombe*. The media did not seem to interfere with the experiment. Similarly, the selection marker did not interfere as the *ctk1::kanR* strain was as sensitive as the wild type. The deletion of *ncs6*, which results in the absence of the s² moiety of the double modification resulted in a reduced sensitivity to zymocin compared to the absence of Elongator.

These results indicate that *S. pombe* is much less sensitive to extracellular zymocin. As indicated above, this may result from differences in cell wall composition that alter the binding

Lys tRNAs

5'probe (3402)	3'probe (3403)	
TCCCGTTTAGCTCAATCGGctTAGAGCGTCCGGCTTTTA.....ACCGGAAG.....GtTGCAGTTCGAGTCTCGCAGTGGGAG		tRNA-TTT modified
TCCCGTTTAGCTCAATCGGctTAGAGCGTCCGGCTTTTA.....ACCGGAAG.....GtTGCAGTTCGAAATCTCGCAGTGGGAG		
TCCCGTTTAGCTCAATCGGctTAGAGCGTCCGGCTTTTA.....ACCGGAAG.....GtTGCAGTTCGAGTCTCGCAGTGGGAG		tRNA-CTT unmodified
TCCCGAGTGGCTCAATCGGtTtAGAGCGTCTGACTCTTAtgatggtcATCAGAAG.....GtTGCAGTTCGAGTCTCGCCTTGGGAG		
TCCCGAGTGGCTCAATCGGtTtAGAGCGTCTGACTCTTAtgatggtcATCAGAAG.....GtTGCAGTTCGAGTCTCGCCTTGGGAG		
TCCCGAGTGGCTCAATCGGtTtAGAGCGTCTGACTCTTAtgatggtcATCAGAAG.....GtTGCAGTTCGAGTCTCGCCTTGGGAG		
TCCCGAGTGGCTCAATCGGtTtAGAGCGTCTGACTCTTAcgaatggtcATCAGAAG.....GtTGCAGTTCGAGTCTCGCCTTGGGAG		

Glu tRNAs

5'probe (3438)	3'probe (3439)	
TCCGTTGTGGTCCAACGGCtA.GGATTCGTCGCTTTCACCGACGCG.....GTCGGGGTTCGACTCCCCGCAACGGAG		tRNA-TTC modified
TCCGTTGTGGTCCAACGGCtA.GGATTCGTCGCTTTCACCGACGCG.....GTCGGGGTTCGACTCCCCGCAACGGAG		
TCCGTTGTGGTCCAACGGCtA.GGATTCGTCGCTTTCACCGACGCG.....GTCGGGGTTCGACTCCCCGCAACGGAG		tRNA-CTC unmodified
TCCGTTGTGGTCCAACGGCtA.GGATTCGTCGCTTTCACCGACGCG.....GTCGGGGTTCGACTCCCCGCAACGGAG		
TCCGTATGGTCCAAGTGGCtA.GGATTCATCGCTCTACCGATGCG.....GCGGGGGTTCGATTCCCCCTGACGGAG		

Figure 14. Alignment of each Glu tRNA and Lys tRNA. The sequence corresponding to the different tRNAs are aligned. The first sequence is the sequence which serves as template for the probe. The green characters correspond to a correct alignment and red ones correspond to a nucleotide difference. The blue lines indicate the site of liaison for the different probe. The orange lines separate the tRNA modified by Elongator and the tRNA unmodified.

of zymocin or its entry in the cell. However, provided that zymocin efficiently enters fission yeast cells, differences in the pool of modified and unmodified tRNAs between budding and fission yeasts may also explain the reduced sensitivity of fission yeast. Taken together, these data do not allow us to conclude that γ -toxin can target the mcm^5s^2 modified tRNAs in fission yeast. We therefore decided to set up a γ -toxin cleavage *in vitro* assay.

1.2. *In vitro* tRNA cleavage with purified GST- γ -toxin

In order to know if γ -toxin is efficient on fission yeast tRNAs, we have tested the activity of purified, GST-tagged, toxin on tRNAs *in vitro* (Figure 13). Based on previous work by the Byström lab, (Huang et al., 2008; Lu et al., 2005), we first expressed and purified a γ -toxin/GST fusion in *E. coli*. The construction was generated by the restriction of pDH461 with BamHI and XhoI enzymes followed by ligation of the γ -toxin ORF amplified with primers containing the corresponding enzymes restriction sites. After transformation in the proper *E. coli* expressing strain (BL21), the one-step purification protocol of GST- γ -toxin resulted in highly pure fractions at concentration ranging from 0.5 to 1 mg/ml with little signs of degradation.

Using purified tRNAs from a wild type and an Elongator mutated strains as substrate, we set up a tRNA cleavage assay using increasing amount of the GST- γ -toxin while the single GST was used as negative control. The reaction products were resolved on PAGE and probed by Northern blotting using a labelled antisense probe. After several attempts to adjust the method, we obtained our first results with a probe targeting the 5' end of tRNA^{Lys}_{UUU}. The higher bands correspond to the full-length tRNAs and the lower band is the 5' end. While the increased amount of γ -toxin correlated with increased cleavage, the effect remained marginal with the bulk of tRNAs being unaffected. In addition, a similar effect was noted when tRNAs from an Elongator mutant were used as substrate. The Byström team reported that the requirement of the modification for cleavage is bypassed by higher concentration of γ -toxin but we did not observe any difference between wt and mutant even at the lowest dose that would still generate cleavage. The low efficiency of the cleavage and the apparent uncoupling from the presence of the modification led us to repeat and improve the assay.

We repeated the experiment with probes targeting the 5' and the 3' ends of tRNA^{Gln}_{UUG} as this tRNA was shown to be the most efficiently targeted by γ -toxin (Lu et al., 2005). While we initially planned to use both the 5' and the 3' probes, technical issues led us to use the 5' probe for the wt and the 3' probe for the mutant. While this is obviously not ideal and could not be resolved due to lack of time, it should not influence the result as the two tRNA ends behave identical. We observed again two bands corresponding the full-length tRNAs and cleaved tRNAs respectively. For samples from the wild type, a reverse gradient in the intensity of full-length and cleaved bands, correlating with the increased amount of GST- γ -toxin used, was observed. When 5 μ g of GST- γ -toxin was used, nearly the entire pool of tRNA^{Gln}_{UUG} was cleaved. By contrast, the pool of tRNA^{Gln}_{UUG} from the Elongator mutant was mainly resistant to increased amount of GST- γ -toxin. It could be that a subset of thiolated tRNAs is still present and processed by GST- γ -toxin. The amount of toxin may not be limiting in that case and that this pool is entirely cleaved at the lowest concentration of toxin used. Unfortunately, as the probe used to reveal tRNA^{Gln}_{UUG} cleavage in wild type and *elp3* deleted strains are different, we cannot compare the intensity of bands. Indeed, the difference could be the result of a difference probe affinity for tRNA^{Gln}_{UUG}.

Taken together, these experiments indicate that tRNAs from fission yeast are likely sensitive to cleavage by γ -toxin in a modification-dependent manner. It is difficult however to conclude anything about the efficiency of the cleavage. Moreover, there are 13 copies of the tRNA^{Lys} in the fission yeast genome, among which only four harbour the UUU anticodon and the rest with

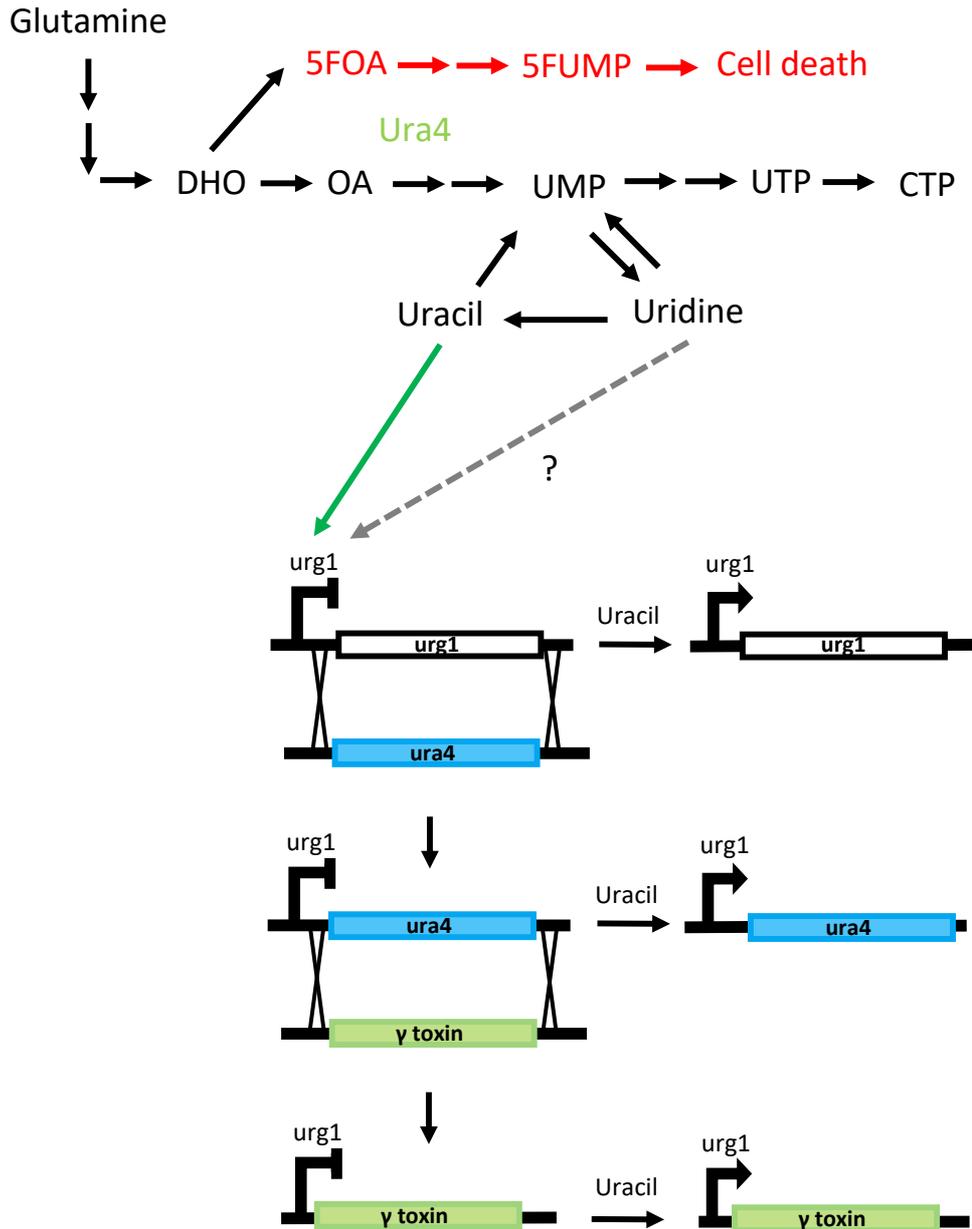


Figure 15. The *ura4/5-FOA* system at *urg1* locus. The *ura4* gene encodes for a protein implicated in the pathway generating CTP. The addition of 5FOA in presence of Ura4 leads to the cell death due to the generation of a toxic metabolite (5FUMP). This system allows to select integrations replacing the *ura4* gene in a strain auxotroph for uracil. For the integration of toxin, the addition of uracil is forbidden as it activates the promoter controlling the toxin. It is possible to replace the uracil by uridine used in the same way by yeast in absence of *ura4* gene. It is not known if the activation of *urg1* promoter is possible with uridine. DHO, dihydroorotate; OA, orotic acid; UMP, Uracil monophosphate, UTP, uracil triphosphate; CTP, cytosine triphosphate; 5FOA, 5 fluoroorotic acid; 5FUMP, 5 fluorouracil monophosphate.

alternative CUU anticodon (Figure 14). Considering that the sequence of these tRNAs is very similar, the probes could recognise the whole pool of modified and unmodified species. In addition, as Elongator can be regulated, the pool of modified tRNAs could depend on environmental conditions, adding further complexity to the analysis.

In conclusion, these experiments suggest that even if zymocin is inefficient at counteracting *S. pombe* growth when secreted by *K. lactis*, purified GST- γ -toxin can cleave purified tRNAs from fission yeast, likely in a modification-dependent manner. The tRNA^{Gln}_{UUG} is the preferred substrate like for budding yeast. We therefore decided to pursue the project by constructing a strain that would allow regulated expression of γ -toxin *in vivo* in fission yeast.

2. Regulation of the *urg1* promoter in fission yeast

The *urg1* promoter is quickly and strongly induced by the addition of uracil to the culture. In the frame of a screening strategy, this is a big advantage as it combines the use of a cheap reagent with the facility of the induction. However, the integration of the *K. lactis* gene encoding the γ -toxin downstream of the *urg1* promoter requires the use of a 5-FOA selection. First, the *urg1* gene is replaced by the *ura4* gene, which allows the growth of integrators on a media lacking uracil, provided that an uracil auxotroph strain is used. Secondly, the *ura4* gene is replaced by the γ -toxin gene and the event is selected on 5-FOA, a compound which is rendered toxic by the Ura4 enzyme encoded by the *ura4* gene. This two-step positive selection is the gold standard in yeast genome engineering. Because exogenous uracil must be provided to strains lacking *ura4*, the problem is that this will activate the *urg1* promoter, which should lead to the induction of γ -toxin (Figure 15). Interestingly, yeast can use uridine rather than uracil to ensure growth when *ura4* is absent. In fact, uridine is often used as it is far more soluble than uracil and therefore easier to use. However, the effect of uridine on the *urg1* promoter was never tested. We therefore performed a RTqPCR and a Northern blot to test this.

We first performed a pilot (one replicate) time course RTqPCR analysis on a wt strain after addition of either uracil or uridine both at 0.25 mg/mL (Figure 16). Cell pellets were collected at 0, 15, 30 and 60 minutes with the time 0 sample collected straight after addition of the reagent. After 15 minutes, the induction of *urg1* reached a fold change of 7 followed by a slight settlement after 30 minutes and maximal induction after 60 minutes. In contrast, the addition of uridine showed no increase of fold change for any timing, indicating that uridine does not induce expression of *urg1* and can therefore be safely used. However, the use of an uracil concentration of 0.25mg/mL implies that the stock solution is quite above its solubility threshold, which makes it difficult to work with. We therefore tested if a lower concentration, of 0.072mg/mL is suitable for induction. In addition, we have tested if the addition of arginine (0.1mg/mL) affected the basal level of the promoter in case of this basal expression would still be enough to generate toxic level of γ -toxin. Arginine was indeed reported to repress *urg1* (Watson et al., 2013). After 15 minutes of induction, both concentrations of uracil show a similar expression level. However, after 30 minutes, a decrease was observed in both cases. Moreover, the higher concentration of uracil led to a re-increase after 60 minutes, contrary to the condition with uracil at lower concentration. Finally, the addition of arginine reduces drastically the expression level of *Purg1*. It needs also to consider that the fold change of expression is lower compared with the first test. This second pilot assay taught us that a lower level of uracil seems to induce *Purg1* as well as higher concentration. In consequence, we have decided to realise a triplicate with uracil at the lower concentration to confirm these data. The results obtained with this experiment are more in correlation with data from Watt et al. (Watt et al., 2008). The fold change already increases after 15 minutes of induction and reaches its maximum after 30 minutes.

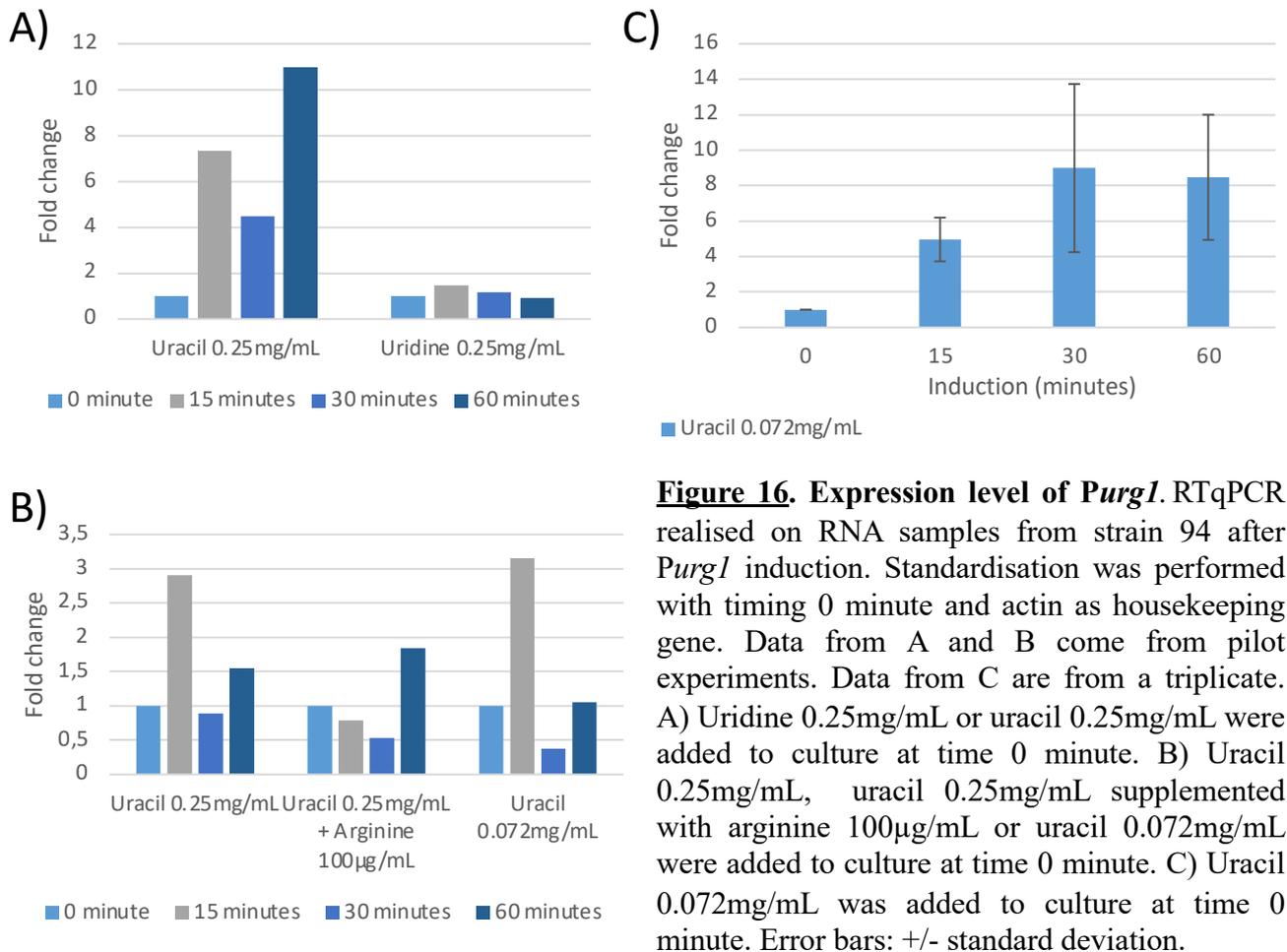


Figure 16. Expression level of *Purg1*. RTqPCR realised on RNA samples from strain 94 after *Purg1* induction. Standardisation was performed with timing 0 minute and actin as housekeeping gene. Data from A and B come from pilot experiments. Data from C are from a triplicate. A) Uridine 0.25mg/mL or uracil 0.25mg/mL were added to culture at time 0 minute. B) Uracil 0.25mg/mL, uracil 0.25mg/mL supplemented with arginine 100µg/mL or uracil 0.072mg/mL were added to culture at time 0 minute. C) Uracil 0.072mg/mL was added to culture at time 0 minute. Error bars: +/- standard deviation.

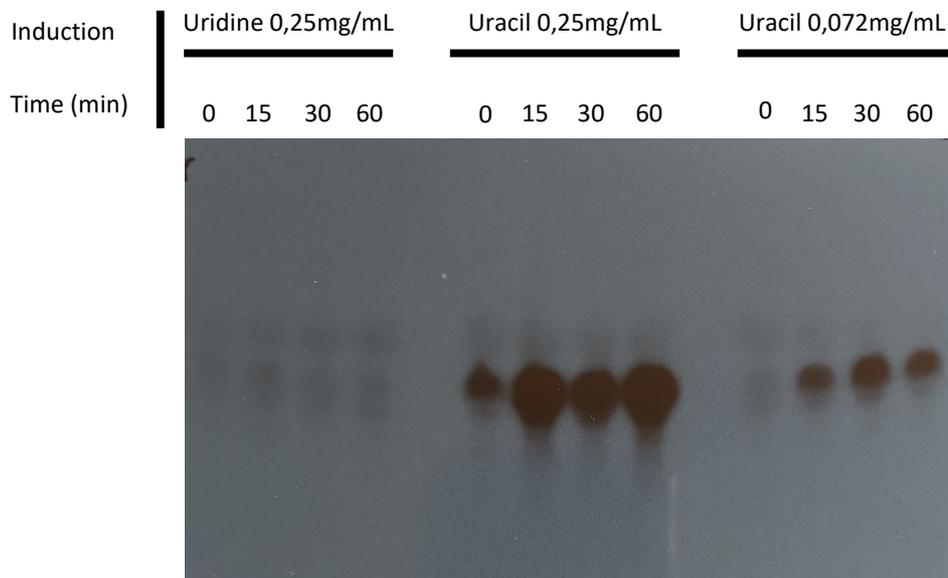


Figure 17. Expression level of *Purg1* with uracil and uridine addition. Northern blot realised on RNA samples from strain 94 after *Purg1* induction. Uridine 0.25mg/mL, uracil 0.25mg/mL or uracil 0.072mg/mL were added to culture at time 0 minutes. The membrane was radiolabelled with α -³²P Random Primers Labeling and exposed for 3 hours.

In order to consolidate these data, we performed a Northern blot with the same RNA samples as RTqPCR and a multiprimed probe corresponding to *urg1* (Figure 17). The experiment unambiguously confirmed that uridine does not induce *Purg1*. It was also clear that the higher uracil concentration led to higher and faster induction. The basal level of the *urg1* mRNA at time 0 was under the detection threshold and the low level of *urg1* at time 0 when uracil was added at 0.25mg/mL likely results from the very fast induction (the sample was centrifugated just after the addition of uracil).

In summary, these data indicate that uracil at 0.072mg/mL can be used to induce *Purg1* while reaching lower expression level than uracil at 0.25mg/mL. This lower induction could be useful in our case as the Bystöm lab had already shown that γ -toxin at high concentration is also able to cleave unmodified tRNAs, at least *in vitro* (Lu et al., 2005). Secondly, the addition of uridine in media allows growth of *ura4*-D18 strains without inducing *Purg1*. Finally, the Northern blot indicates that the basal expression of *urg1* is very low compared to the expression level after induction, suggesting that basal level should not be a problem in the case of γ -toxin. All these data are encouraging and reinforce the choice of *Purg1* as inducible promoter for γ -toxin expression.

3. Integration of the γ -toxin encoding gene at *urg1* locus

The biggest part of the project was to integrate the gene of the toxin into the fission yeast genome. An advantage of yeast is the facility to integrate a gene thanks to the high homologous recombination level occurring naturally in this model organism. Moreover, the haploidy of yeast allows to generate mutants with only one copy of the gene. However, as γ -toxin is expected to be lethal for yeast expressing Elongator, it was wise to attempt constructing the strain in a wt and an Elongator deletion background.

3.1. Integration of γ -toxin by the *ura4*/5-FOA system

We first attempted to integrate the gene of γ -toxin at the *urg1* locus using the classical *ura4*/5-FOA double selection (Figure 18.A). As already mentioned, 5-FOA is metabolized in presence of the *ura4* gene encoding orotidine 5'-phosphate decarboxylase to generate a toxic metabolite, the 5-fluorouracil, which induced cell death. Two *ura4*-D18 strains, a wild type (strain 96) and a strain deleted for *elp3* (strain 674) were used to replace the *urg1* ORF by the *ura4* gene. The two newly generated strains, 2060 and 2061, wild type and *elp3::natR* respectively were selected on minimal media lacking uracil and confirmed to have the right genotype by PCR (Figure 18.B). In a second step, the γ -toxin gene flanked by homology arms targeting the *urg1* locus was transformed and the replacement of *ura4* was monitored by the ability to grow on a media supplemented with 5FOA. At this step, uridine is added as the expected strains will return to uracil auxotrophy. However, the problem is often background growth as a competition between 5-FOA and uridine occurs. The problem persisted even after doubling the concentration of 5-FOA in the media (from 1mg/mL to 2mg/mL). The few isolated clones came back negative from genotyping by colony PCR verification. Therefore, the method appeared inefficient at targeting the γ -toxin gene at the *urg1* locus. The *urg1* gene is not characterized but likely connected to the uracil metabolism as its expression is strongly induced by this compound. Therefore, it is possible that its deletion alters the sensitivity to 5-FOA, rendering the classical system of integration inefficient. This led us to consider an alternative approach.

3.2. Integration of γ -toxin-kanR gene

In a second attempt to target the γ -toxin gene to the *urg1* locus, we constructed an integration cassette where the gene is followed by the kanR selection marker (Figure 19). The γ -toxin ORF was amplified by PCR using primers containing the NdeI and AscI restriction sites and the PCR

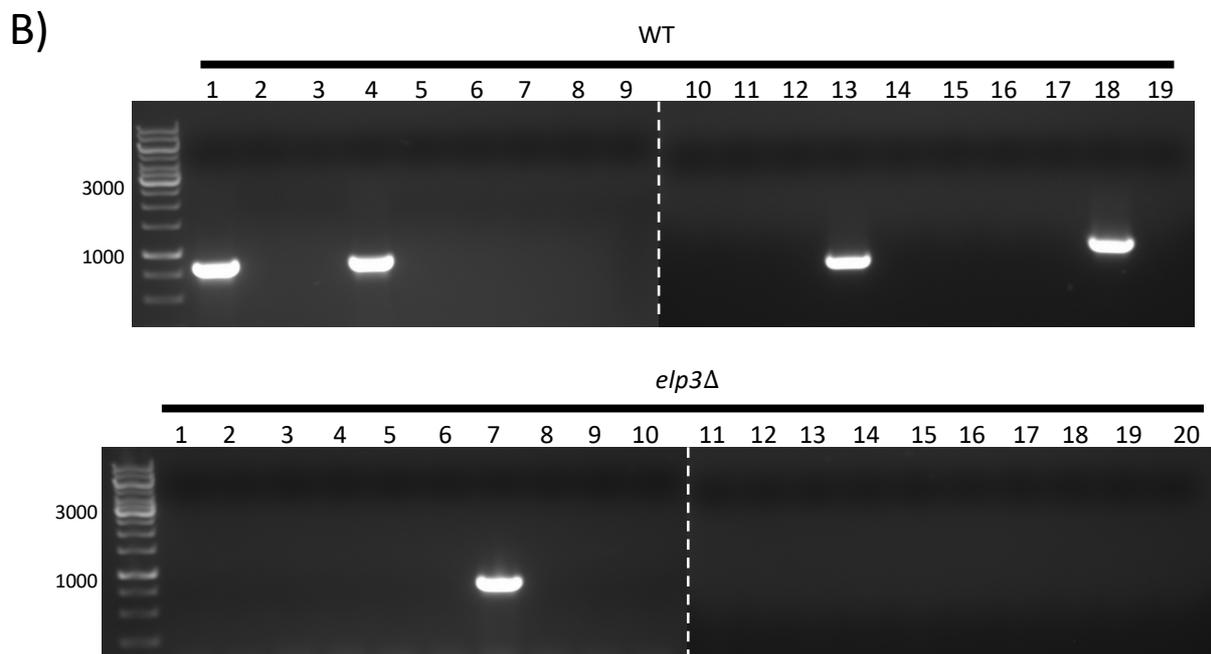
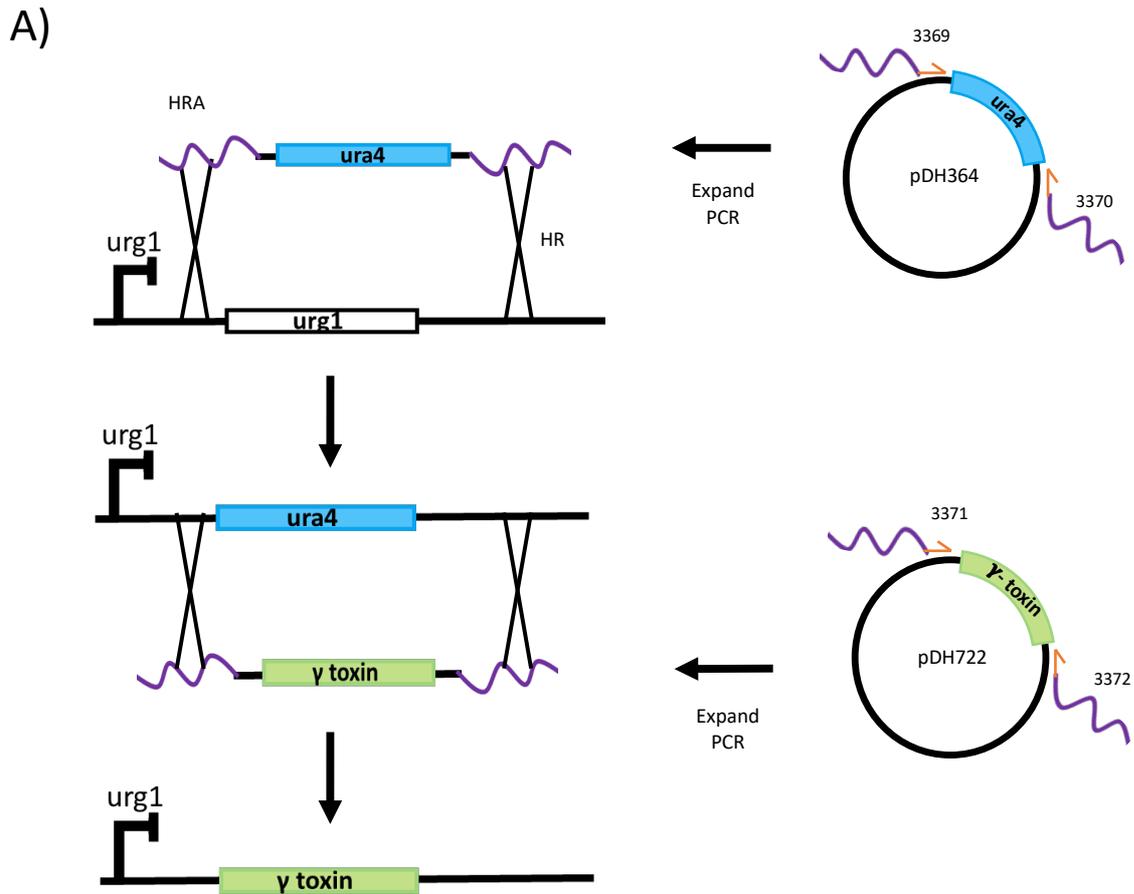


Figure 18. : Integration of γ -toxin after *urg1* deletion by *ura4* at *urg1* locus. A) The integration of *ura4* gene encoding is performed by homologous recombination a locus *urg1*. The integration of the γ -toxin gene is targeted to the *urg1* locus by homologous recombination after *ura4* integration. This double step allows the selection of correct γ -toxin integration thanks to 5FOA. The integrated DNA sequences were generated by expand PCR with primers and plasmids indicated. B) Gel migration to check the integration of *ura4* gene at *urg1* locus. The integration was made in the strain 96 (wt) and 674 (*elp3* Δ) and control with colony PCR amplification (primers 3270 and 741). PCR product length: 825pb. HR: Homologous recombination. HRA: Homologous recombination arm

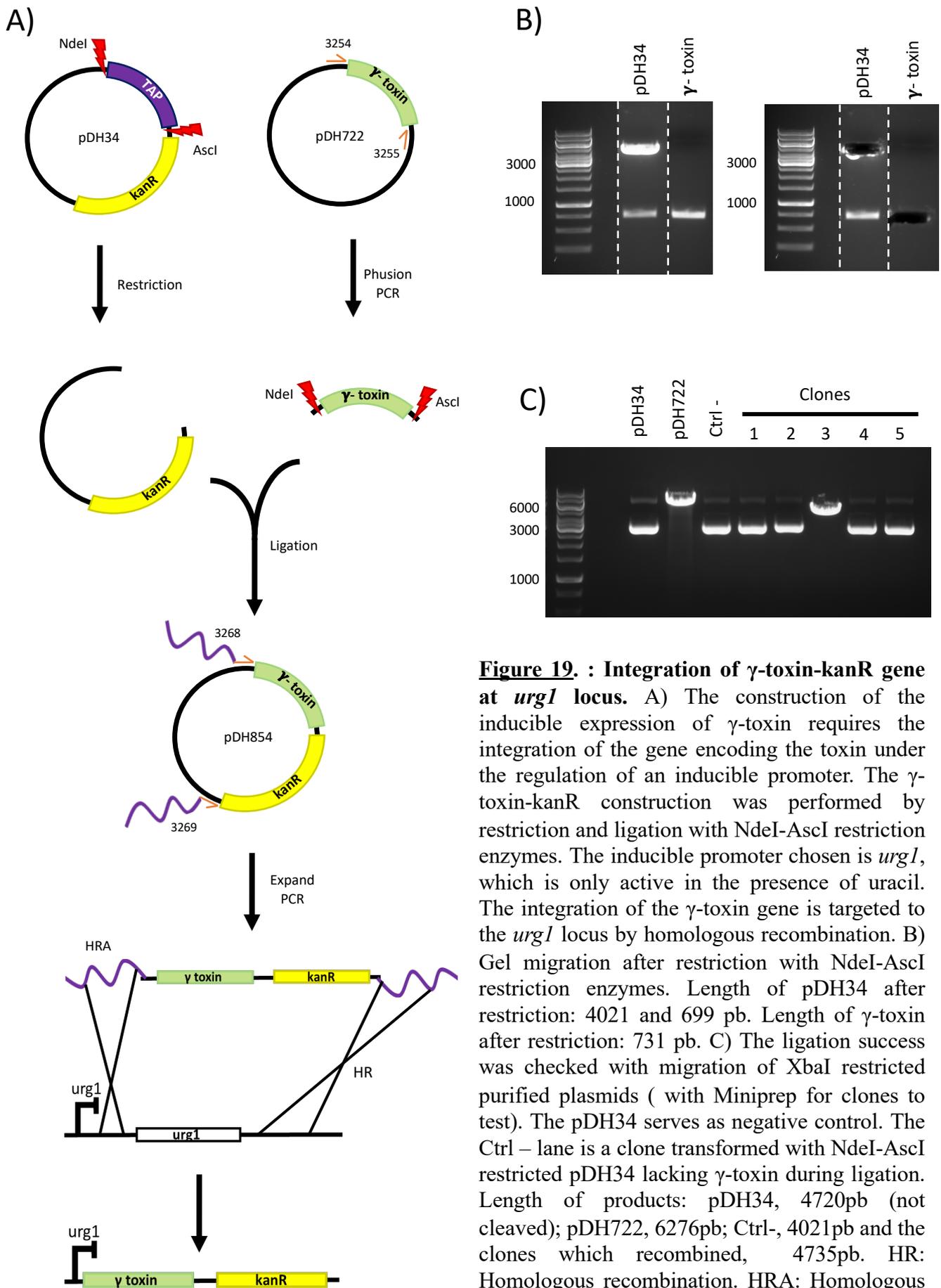
product was ligated in the pDH34 plasmid digested with the corresponding enzymes. The presence of the geneticin antibiotic should allow us to select the positive clones that have integrated the cassette. Several attempts of transformation never resulted in any positive clones. In view of these results, we assessed a colony PCR expanding *urg1* to ensure that problem did not come from primers used for colony PCR targeting γ -toxin-kanR. However, every clone tested were positives for *urg1* showing that problems came for recombination (Figure 20.A). We next checked the integration rate at the locus *urg1* to test the efficiency of integration at this locus. Indeed, the homologous recombination rate is known to strongly vary depending of the locus likely because of nucleosome density. We tried to replace the *urg1* gene with the kanR selection marker alone (Figure 20.B). On 16 clones tested per strain, 3 were positives for the wild type and 3 for the *elp3* Δ (Figure 20.C). Therefore, a 19% success rate was obtained independently of the genotype, which does not seem unusual. A hypothesis explaining the failure to integrate the γ -toxin gene-kanR cassette compared to the kanR cassette alone is that the longer cassette can circularize or adopt structures preventing its integration in the genome.

3.3. Integration of γ -toxin with CRISPR-Cas9 method

Following failures of integration using 5-FOA based procedures, we have decided to test another method based on CRISPR-Cas9 cleavage. We have followed the protocol developed by Zhang *et al* (Figure 21). The method consists in a transformation with a DNA mix composed of a pre-digested plasmid encoding for Cas9, a PCR product corresponding to the guide RNAs designed to recognise a specific region of the yeast genome and a repair sequence. Due to homology regions between the linearized Cas9 plasmid and the PCR product, yeast cells naturally repair the plasmid through recombination (gap repair), which allows expression of Cas9 and the guide from the same vector without any *in vitro* ligation step (Zhang et al., 2018). In the present case, two sgRNAs were designed to target the *ura4* gene integrated at *urg1* locus in strain 2060 and 2061. As the genuine *ura4* locus is deleted, Cas9 could not cleave elsewhere than at *urg1* locus. The repair DNA contained the γ -toxin gene flanked by homologous regions to the *urg1* locus. In addition, the plasmid contains the blasticidine resistance marker allowing the selection of positive clones. Positives clones were next plated on media lacking blasticidine to relieve the selective pressure and allow loss of the Cas9 plasmid.

Before genotyping by PCR, clones were plated on different media supplemented or not with uridine or 5-FOA in order to assess the status of the *ura4* gene. Every clone of the *elp3* deleted strain were tested by PCR but none were positive for γ -toxin gene integration. A single clone of the wild type strain (2060) could grow in the presence of 5-FOA and was positive in PCR probing the presence of the γ -toxin gene at the *urg1* locus. Sequencing confirmed its presence. Why the integration of the γ -toxin gene at the *urg1* locus is so difficult is unclear. The locus did not appear particularly difficult in control integrations. It could be that γ -toxin is effective even at very low dose. However, the transformation into an *elp3* deleted strain that is expected to be resistant to the toxin should have prevented this problem and yet was not more successful.

We next analysed the effect of uracil on the *Purg1::* γ -toxin strain (Figure 22). Surprisingly, the presence of uracil did not affect growth, indicating that the induction of the *urg1* promoter was either inefficient or that the presence of γ -toxin did not affect cell growth. RTqPCR analyses support the first hypothesis as no increase of the level of the γ -toxin mRNA was noticed upon uracil addition while the *urg1* was properly induced in the control strain. This is very unexpected as the only difference between these two strains is the exact replacement of the *urg1* ORF by the γ -toxin ORF. This data led us to test the induction of the γ -toxin mRNA under the control of another promoter. Considering the above-mentioned difficulties, we decided to work with plasmids and added an HA tag to monitor protein expression.



4. Expression of γ -toxin under *nmt* promoter

The *nmt* (no message in thiamine) promoter series offers an alternative as switch off promoters in fission yeast. The presence of thiamine strongly represses the *nmt* promoters that exist in three versions: *nmt1* (strong activity), *nmt41* (intermediate activity) and *nmt81* (low activity) covering a large range of expression levels. We amplified the γ -toxin ORF by PCR with primers harbouring the Sall and BamHI restriction sites and cloned it in the appropriately digested pREP plasmids (pREP1, pREP41 and pREP81) to place γ -toxin ORF under the control of P*nmt* of various strength (pDH862, pDH864 and pDH866 respectively). In parallel set of cloning, an HA tag was added in the reverse primer used for PCR in order to generate HA-tagged versions of γ -toxin (pDH863, pDH865 and pDH867 respectively). After transformation, we tested the induction of γ -toxin from these plasmids by a growth assay and a Western blot.

4.1. Physiological response after induction

The physiological response was assessed by a drop assay on minimal media in the presence or absence of thiamine after 22h of growth in liquid minimal media with or without thiamine. The first attempt failed as any strain growth on the plates (Figure 23.A). No difference in growth were observed in any condition, which led us to verify the induction of the γ -toxin protein by western blotting.

4.2. Protein level after induction

We applied the same conditions as above, namely liquid growth in minimal media in the presence or absence of thiamine for 22h (Figure 23). In the first trial, we could not detect any specific signal using an anti-HA antibody. We therefore repeated the experiment with an additional positive control consisting of a pREP41 expressing the Lsk1-HA protein previously constructed in the lab. A band at the predicted 70kDa was detected upon induction in the positive control. However, no specific band corresponding to the predicted size of γ -toxin-HA was detected.

These two experiments indicate that γ -toxin is not produced in fission yeast whatever the strength of the *nmt* promoter used. They also explain why no growth defect was noted upon induction. A hypothesis is that the expression of γ -toxin is toxic for the cells, leading to a death or a slow growth. The consequence could be the selection of cells that have less or truncated plasmids, leading to a growth advantage. At this stage, we have not checked the induction at the transcriptional level and cannot further comment on this. However, the analysis of the different sequences of our plasmids highlighted the presence of an out of frame ATG just before the genuine ATG of γ -toxin. A possibility is that this ATG is recognised in place of the normal ATG, resulting in the expression of an aberrant peptide. (We removed this ATG by *in vitro* mutagenesis and repeated the experiment).

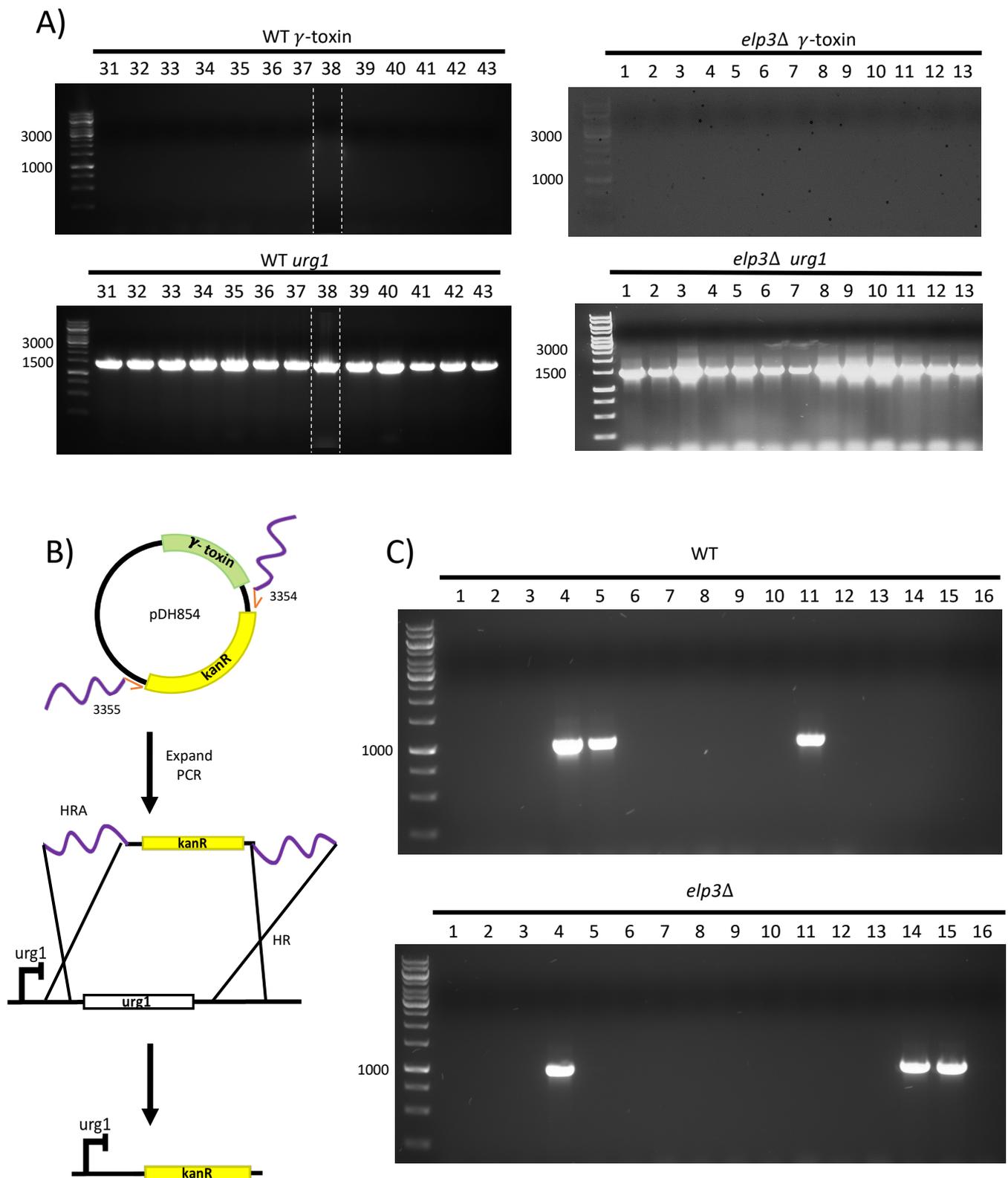


Figure 20. : Integration of *kanR* gene at *urg1* locus. A) Verification of the integration of the γ -toxin gene in strain 94 (wt) and 2054 (*elp3Δ*) by amplification of γ -toxin (3270-3255) or *urg1* (3270-3306) at *urg1* locus. PCR product length: 900 pb for γ -toxin and 1500 pb for *urg1*. B) The integration of *kanR* gene at *urg1* locus was performed to check the integration rate at *urg1* locus. C) Verification of the integration of the *kanR* gene in strain 94 (wt) and 2054 (*elp3Δ*) by amplification of *kanR* (3270-3356) at *urg1* locus. PCR product length: 1000 pb for *kanR*. HR: Homologous recombination. HRA: Homologous recombination arm.

Conclusion and perspectives

I would like to discuss four important issues raised during this work and important for the rest of the project.

First, my data show that the *Purg1* is only inducible by the addition of uracil and that the strength of induction is dependant of the concentration of uracil used. Uridine that is used to ensure the growth of auxotroph mutants harbouring the *ura4-D18* allele has not detectable effect of *urg1*. The data also indicate that arginine could be useful to repress the expression of the promoter in case of the “OFF-state” would still allow the expression of enough toxin to kill the cells. Despite many difficulties, we obtained a strain expressing γ -toxin gene under control of *Purg1*, which opens the possibility of various experiments to assess the efficiency of our read-out system. The first attempt to induce γ -toxin is disappointing as not cell death is observed. The integrated sequence at the locus was checked to be correct and the only difference with the wild type strain is therefore the replacement of the *urg1* ORF by the ORF of the toxin. At the molecular level, no induction of the toxin mRNA was detected, which fits with the absence of lethality. It could be interesting to test the induction of *Purg1* after the replacement of *urg1* gene by another gene to understand where the induction problem comes from. We note that the γ -toxin is extremely enriched for A/T as previously noted by others (Schaffrath and Meinhardt, 2004). While this did not impede expression in a *K. lactis* related species (namely *S. cerevisiae*), it could be that fission yeast is more sensitive to this bias at either the transcriptional level, the translational level or both. A way to address this issue would be to alter the A/T content while preserving the coding to reach the average observed in fission yeast. We also have to keep in mind that only one positive clone was recovered when targeting the γ -toxin ORF to the *urg1* locus. While this is in theory not a problem, we cannot completely exclude that the positive clone has an additional mutation that allowed the integration. In that context, it is possible that even very low level of γ -toxin is lethal in the absence of this extragenic mutation. This is at this stage pure speculation though and full genome sequencing would be necessary to address this issue. In order to complete the analysis of the generated strain, HA-tagging is in progress in order to measure the level of the γ -toxin protein produced.

Second, the present work brought information about the ability of γ -toxin to target *S. pombe* tRNAs. While it is clear that zymocin is inefficient as killing fission yeast when secreted from *K. lactis*, we detected cleavage activity *in vitro* when using the GST- γ -toxin we have purified. Our data indicate that at least *in vitro*, a cleavage independent of the concentration of GST- γ -toxin is observed when using tRNAs purified from an Elongator mutant. It is possible that the remaining thiol is sufficient to provide basal activity. Previous work from the lab has shown that thiolation of dependent on the preliminary synthesis of the mcm⁵ chain and the pool of solely thiolated tRNAs is therefore low. By contrast, when tRNAs purified from wild type are used as substrate, a dose-dependent cleavage is observed with the pool of tRNAs gradually processed in a detected cleaved (and therefore smaller) fragment. Altogether, these data suggest that recombinant γ -toxin targets fission yeast tRNAs with a likely strong preference for the modified tRNA^{Glu}_{UUC}. While encouraging, these data will have to be confirmed *in vivo* when the strain expressing γ -toxin will become available.

Third, an important issue is the fact that some species are resistant to the expression of γ -toxin *in vivo* due to the presence of a ligase that repairs the cleaved tRNAs. Elegant work in budding yeast has proven that the expression of the corresponding plant ligase is sufficient to provide immunity to budding yeast (Nandakumar et al., 2008). While *S. cerevisiae* possesses an orthologue of the ligase, it lacks one domain likely responsible for the repair of the γ -toxin lesion. Interestingly, the *S. pombe* orthologue is similar to the *S. cerevisiae* enzyme. We

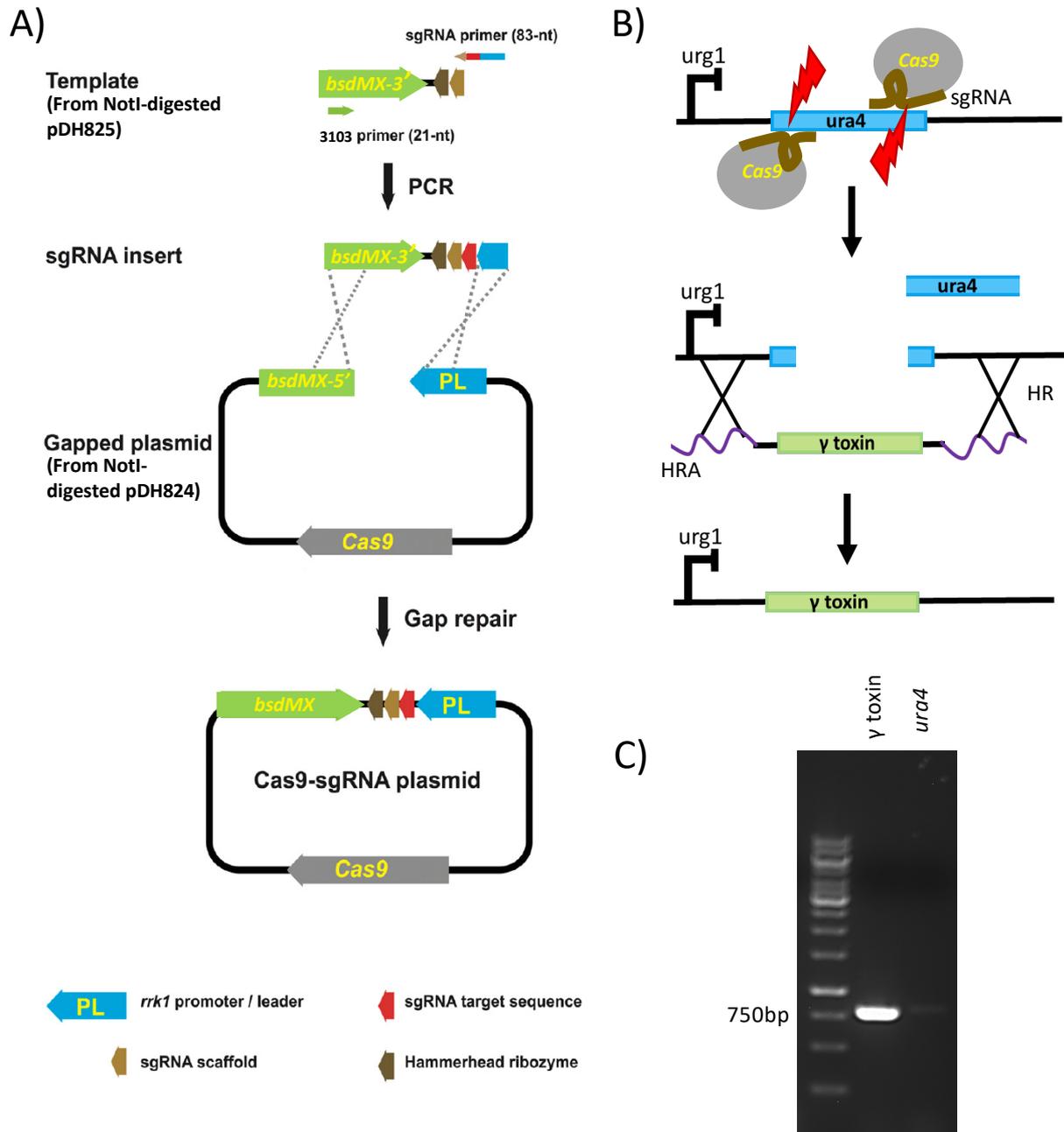


Figure 21. Integration of γ -toxin with CRISPR-Cas9. CRISPR-Cas9 system is composed by NotI digestion of pDH824 and pDH825. The pDH825 serves as template for the two sgRNAs. The pDH824 is the plasmid with Cas9 coding sequence. Adapted from (Zhang et al., 2018). B) The Cas9 cleaves specific site thanks to both sgRNAs. The integration of the γ -toxin gene is targeted to the *urg1* locus by homologous recombination enhanced by the double strand breaks generate with CRISPR-Cas9 method. This double step allows the selection of correct γ -toxin integration thanks to 5FOA. C) Colony PCR amplifying 750pb of γ -toxin and *ura4* genes at *urg1* locus of the only positive clone of the CRISPR-Cas9 transformation in strain 2060 (wild type). HR: Homologous recombination. HRA: Homologous recombination arm. sgRNA: single guide RNA

therefore anticipate that the possibility of repair is very unlikely in fission yeast and will not be a problem in the present case.

Four, considering the difficulties discussed above, we wanted to develop a system to titrate the level of expression of γ -toxin in fission yeast and analyse the phenotypic response. We used the fission yeast pREP system that relies on the thiamine-repressed *nmt* promoter. The interesting feature of this system is that three vectors exist (pREP81, pREP41, pREP3) with different TATA box. Therefore, a full range of expression levels from barely detectable (pREP81-OFF) to very high (pREP3-ON) is available. We cloned the γ -toxin ORF in the corresponding plasmids in order to express either the genuine γ -toxin or a C-term HA tagged version. Surprisingly, no expression was detected in any conditions while the HA antibody was confirmed to work. While this may simply reflect that similar issues raised when using the *urg1* promoter also apply here, we noted the presence of an out-of-frame ATG present in the vectors upstream of the ATG of the γ -toxin ORF. Site-directed mutagenesis of the codon is under way to test if this is the cause of the failure to express γ -toxin from the *nmt* promoter. It should be noted that the *nmt* promoter as such is not ideal in the frame of a screening as its induction relies on the removal of thiamine, which is difficult at large scale. In addition, the intracellular thiamine pool is only depleted after about 20 hours of growth, which renders the induction very slow. However, an alternative of the *nmt* promoter precisely addressing these issues was recently developed by Kjærulff *et al.*, who combined the *nmt* promoter with the *E. coli lacO/lacI* repression system. The resulting plasmid includes a *lacO* operator flanking the *nmt* promoter TATA box and the *lacI* ORF under the control of the strong viral SV40 promoter. The system was reported to lead to fast and high expression in the presence of IPTG (that inhibits LacI) and strong repression by LacI in otherwise wild type medium. Neither the LacI, nor the IPTG inducer disturb the cell. However, the level of expression is reported to be lower than the strongest version of the *nmt* promoter (Kjærulff and Nielsen, 2015). This plasmid, which we recently received is a new option to express γ -toxin and seems interesting for us.

I would like to finish by discussing with some more general issues.

Regardless the promoter used, in case of a really high expression of γ -toxin, the toxin may lead to the cell death even in the absence of the Elongator-dependent modification. It is the reason why it could be needed to find a way to decrease the expression of the toxin. A possibility is to add a DSR degradation motif to the sequence of γ -toxin to reduce its expression. The DSR motif (Determinant of selective removal) is recognized by the Mmi1 protein. It leads to the increased degradation of the DSR-containing mRNAs through the nuclear exosome. This system was elaborated by Watson *et al.* and showed interesting expression level decreased (Watson *et al.*, 2013). However, we consider it as a last resort hypothesis at this stage.

With more time available, it would be useful to repeat the GST- γ -toxin assay with the entire set of 5' and 3' probes for the three tRNAs bearing the modification. It may also be judicious to purify the target tRNAs from the entire pool to exclude cross-reactivity of the probes with a related, unmodified tRNA species. Moreover, to be more precise, the titration of the GST- γ - and the determination of the specific activity would be useful too. This goes hand in hand with the expanded evaluation of enzymatic activity of GST- γ -toxin.

Finally, the possibility that the amount of modified tRNAs among the entire pool of tRNAs varies depending to conditions should be kept in mind. We basically do not know the percentage of modified molecules for a given tRNA. In that context the lab has recently reported that the nitrogen source and the level of TORC activity directly impacted the activity of Elongator (Candiracci *et al.*, 2019). These data provide possible paths to expand our work further.

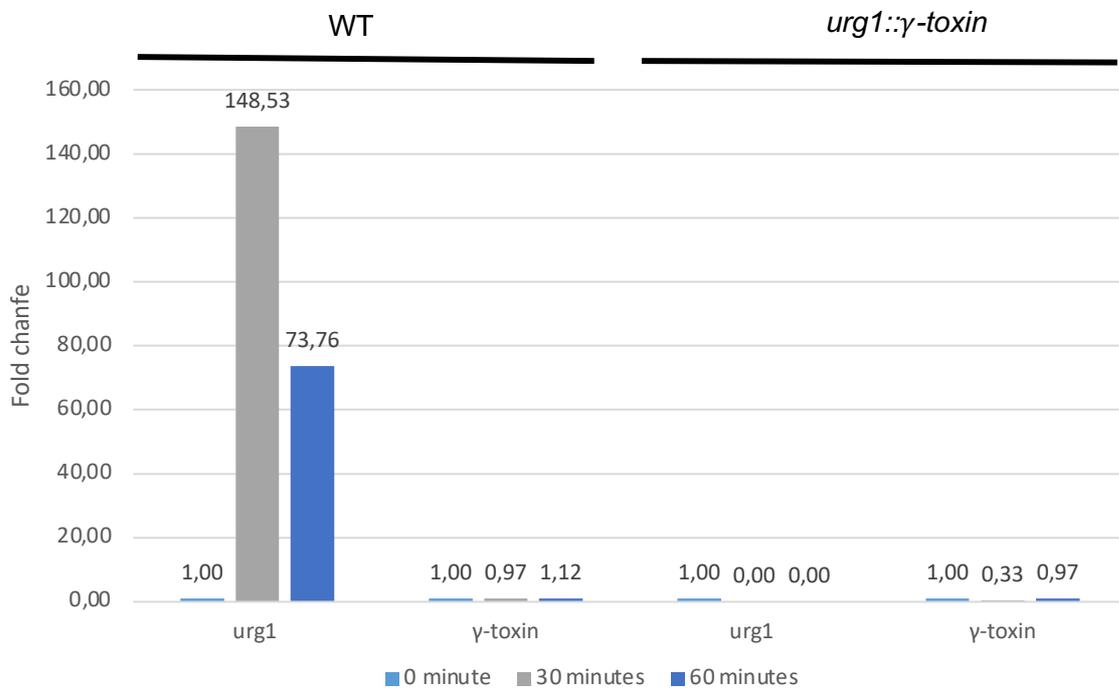
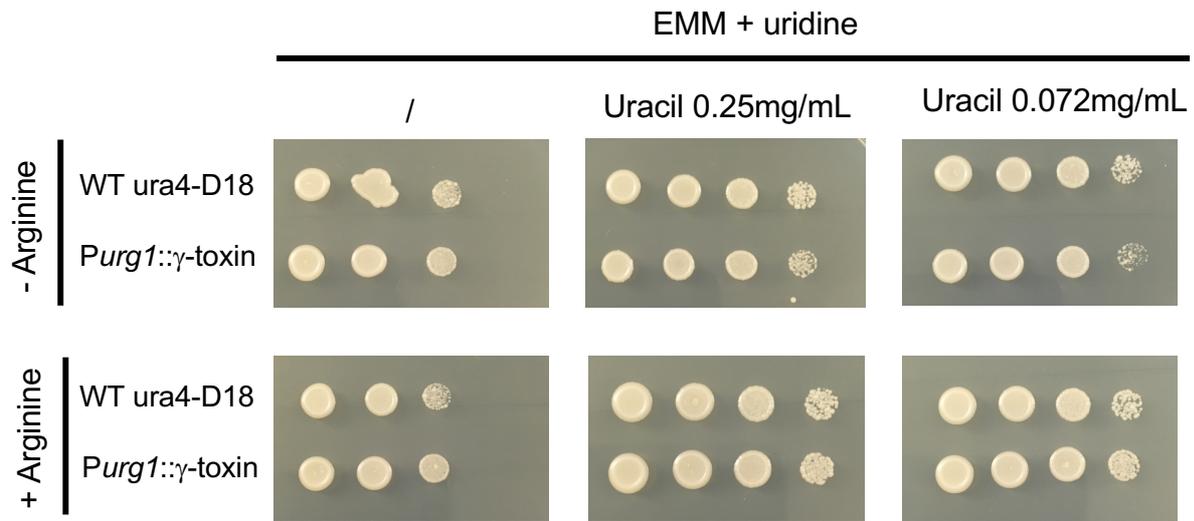


Figure 22. Expression level of *Purg1::γ-toxin* after uracil induction. A) Drop test of four dilutions realised on EMM media supplemented with uridine (75µg/mL) and in the corresponding cases with arginine (100µg/mL). The induction of *Purg1::γ-toxin* was made with the indicated concentration of uracil. The strains used are the strain 96 (WT) and the strain 2074 (*urg1::γ-toxin*). B) RTqPCR realised on RNA samples from strain 96 and 2074 after *Purg1* induction. Standardisation was performed with corresponding timing 0 minute and actin as housekeeping gene. Uracil 0.25mg/mL was added to culture at time 0 minute.

Taken together, the development of an efficient read-out for screening turned out more difficult than anticipated. It will be essential to solve all the issues discussed above before proceeding to the screening.

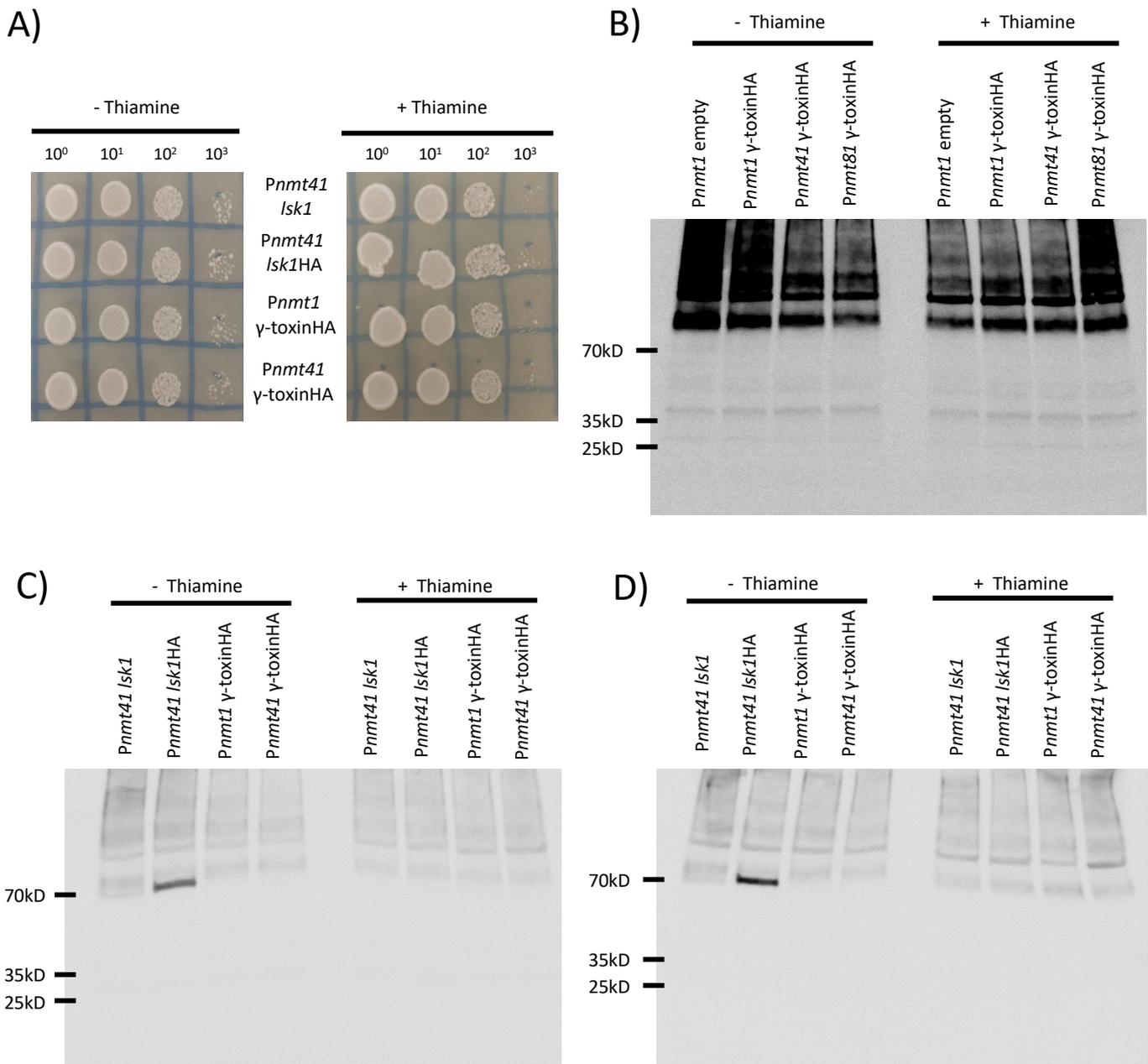


Figure 23. Expression of γ -toxin-HA under control of *Pnmt*. A) Drop test realised on EMM with or without thiamine. The yeasts used are the strain 13 (WT) transformed with pDH79 (*Pnmt1* empty), pDH863 (*Pnmt1* γ -toxin-HA), pDH865 (*Pnmt41* γ -toxin-HA) and pDH867 (*Pnmt81* γ -toxin-HA) after 22 hours without thiamine (for induction) or with thiamine (for control). B) Western blot realised on NaOH extracted protein samples from strain 13 (WT) transformed with pDH79 (*Pnmt1* empty), pDH863 (*Pnmt1* γ -toxin-HA), pDH865 (*Pnmt41* γ -toxin-HA) and pDH867 (*Pnmt81* γ -toxin-HA) after 22 hours without thiamine (for induction) or with thiamine (for control). The membrane was exposed 6 minutes. C) Western blot realised on NaOH extracted protein samples from strain 13 (WT) transformed with pDH596 (*Pnmt41* lsk1), pDH621 (*Pnmt41* lsk1-HA), pDH863 (*Pnmt1* γ -toxin-HA) and pDH865 (*Pnmt41* γ -toxin-HA) after 22 hours without thiamine (for induction) or with thiamine (for control). The membrane was exposed 1 minute 30. D) Western blot realised on NaOH extracted protein samples from strain 13 (WT) transformed with pDH596 (*Pnmt41* lsk1), pDH621 (*Pnmt41* lsk1-HA), pDH863 (*Pnmt1* γ -toxin-HA) and pDH865 (*Pnmt41* γ -toxin-HA) after 24 hours without thiamine (for induction) or with thiamine (for control). The membrane was exposed 1 minute. Primary antibody: rabbit anti-HA. Secondary antibody: anti-rabbit coupled with HRP. Molecular weight of γ -toxin-HA: 28.9kDa. Molecular weight of Lsk1-HA: 65.5kDa.

Material and methods

Yeast strains and growth conditions

The different yeast strains used are listed in table 1 for *S. pombe* and for *S. cerevisiae*. The strain used for *K. lactis* is the IG02. Wild-type and mutant strains of *S. pombe* were grown at 32°C on petri plates or in liquid medium. For liquid culture, the different media used were: YES (Yeast Extract Supplemented: 5g Yeast extract, 30g Dextrose, 0.05g Adenine, 0.05g L-Histidine, 0.05g L-Leucine, 0.05g L-Lysine HCl, and 0.05g Uracil for 1liter of medium from ForMedium), EMM (Edinburgh minimal media from MP Biomedicals) or EMM-N (EMM lacking nitrogen sources from MP Biomedicals) with glutamate (168.8mg/mL) as nitrogen source. In case of petri plates, agar (Difco Agar granulated, BD Biosciences) was added at 2g/100mL of medium. For mating, yeasts were grown in ME (Malt Extract from BD Biosciences). Wild-type and mutant strains of *S. cerevisiae* were grown at 32°C on petri plates of YPD (Yeast Extract peptone dextrose from BD Biosciences). All of these media were supplemented with several components depending on experimentation described further. Adenine was used at 75µg/mL. Leucine was used at 250µg/mL. Arginine was used at 100µg/mL. Uracil was used at 18µg/mL. Uridine was used at 75µg/mL. Thiamine was used at 5µg/mL. 5FOA was used at 2mg/mL. G418 (Geneticin abbreviated Kan) was used at 0.2mg/mL. Nourseothricin (Nat) was used at 0.1mg/mL. Blasticidine was used at 30µg/mL. For some experiments, the specific concentrations needed are indicated.

PCR

Phusion PCR

Phusion High Fidelity DNA Polymerase (M0530S New England BioLabs) were used for amplification of sequences with short primers containing restriction sites. PCR mixes were composed of 5x Phusion HF buffer, 10 mM dNTPs, 10µM forward and reverse primers, template DNA, nuclease free water and 0.2µL of Phusion DNA polymerase (2U/µL) for a total volume of 20µL. The annealing temperature was determined on web site given by manufacturer (www.neb.com/TmCalculator). The extension step last 30 seconds per kb. For loading on gel, 10x *RediLoad* Loading Buffer (750026 ThermoFisher Scientific) was added to sample (2µL for 20µL of final volume).

Expand PCR

Expand High Fidelity PCR System (11732650001 Roche Diagnostics) were used for amplification of sequences with long primers containing homologous sequences for yeast genome. PCR mixes were composed of 10x Expand High Fidelity Buffer (15mM MgCl₂), 20 mM dNTPs, 100µM forward and reverse primers, template DNA, nuclease free water and 4µL of Expand High Fidelity enzyme mix (3.5U/µL) for a total volume of 400µL (4 PCR reactions of 100µL). The annealing temperature was set at 58°C for all Expand PCR.

Colony PCR

GoTaq G2 DNA Polymerase (M748A Promega) were used for amplification of sequences to confirmed gene insertion in yeast genome. PCR mixes were composed of 5x Green GoTaq reaction buffer (M791A Promega), 20 mM dNTPs, 100µM forward and reverse primers, nuclease free water and template DNA for a total volume of 100µL. After 10 minutes of heating at 100°C for cell lysis, 0.5µL of GoTaq G2 DNA polymerase (5U/µL) was added per tube. The annealing temperature was defined by the lower melting temperature of primers.

The protocol provided by the manufacturer was followed for each PCR and the volume of each mix follows these instructions. Plasmids and primers used are listed in table 2 and 3 respectively. All primers had been provided by IDT (Integrated DNA Technologies).

PCR products purification

Phusion PCR

Phusion PCR purifications were performed with Kit MSB Spin PCRapace (1020220400 Invisorb, Stratec molecular). 250 μ L of binding buffer were added to 45 μ L of PCR product and centrifuged 3 minutes at 12000rpm. Purified DNA were eluted with 30 μ L of nuclease free water.

Expand PCR

Expand PCR purifications were performed with QIAquick PCR purification Kit (28106 Quiagen) following manufacturer protocol. Purified DNA were eluted with 30 μ L of nuclease free water.

Colony PCR

Colony PCR purifications were made with Kit MSB Spin PCRapace (1020220400 Invisorb, Stratec molecular) following manufacturer protocol. Purified DNA were eluted with 30 μ L of nuclease free water.

Gel purification

Purifications of PCR products after gel migration were made with QIAquick PCR purification Kit (28106 Quiagen) following manufacturer protocol. Purified DNA were eluted with 30 μ L of nuclease free water.

The quantity of DNA was measured with micro volume spectrophotometer (NanoVue Plus Spectrophotometer GE Healthcare).

Electrophoresis

Electrophoresis were performed on 1% agarose gel. Ladder used was 1% Top Vision LEGQ Agarose (#R0491 Thermo Fisher Scientific). The migration had lasted between 30 and 40 minutes at 140mV. Revelations of gels had been performed with UVP BioDoc-It² Imager (Analytikjena).

Restriction and ligation reactions

Restrictions were realised with FastDigest restriction enzymes (Thermo Fisher Scientific) and certified with gel migration. The reaction mixes were composed of 2 μ L of 10x FastDigest Green Buffer, 1 μ g of plasmid DNA or 0.2 μ g of PCR product, 1 μ L of FastDigest enzyme and water for a total reaction volume of 20 μ L. This mix was incubated at 37°C during time indicated by manufacturers (sometimes a little more to ensure good restriction). Restriction enzymes used were NdeI (#FD0583), AscI (#FD1894), ClaI (#FD0144), XbaI (#FD0684), XhoI (#FD0695), BamHI (#FD0054), NotI (#FD0593), PacI (#FD2204) and SalI (#FD0644). None of these enzymes present star activity unless BamHI which as star activity after more than one hour of incubation.

Ligation were realised with Kit T4 DNA ligase (M180A, Promega). The reaction mixes were composed by 1 μ L of T4 DNA ligase (3U), 2 μ L of 10x buffer, 10 μ L of insert DNA, 5 μ L of vector DNA and water for a final reaction volume of 20 μ L. The mix was incubated at room temperature 3h or at 4°C overnight.

Miniprep

The plasmids were purified with GenElute Plasmid Miniprep Kit (PLN350, Sigma-Aldrich) following the instructions of manufacturer. The quantity of DNA was measured with micro volume spectrophotometer (NanoVue Plus Spectrophotometer GE Healthcare).

Sanger sequencing

All Sanger sequencing were realised by Eurofins Genomics (Mix2Seq Kit).

Bacteria transformation

Bacteria transformations were realised with heat shock method. The transformations were made in *E. coli* DH10b or BL21 competent cells. 100µL of thawed cells were mixed with 20µL of plasmid and incubated on ice for 10 minutes. Heat shock was performed for maximum one minute at 42°C and directly put back on ice. 1mL of LB Broth Base (Lennox L Broth Base Invitrogen 12780-029) was added before incubation of one hour at 37°C. Bacteria were then plated on LB-Amp-agar (Difco Agar granulated, BD Biosciences) and incubate at 37°C overnight or at 25°C for two days for selection. Positives colonies were cultured for Miniprep and diagnostic restriction.

Yeast transformation

The mutagenesis of yeast strains was made by homologous recombination after gene amplification by Expand PCR with primers containing long fragments homologous with yeast genome. 20 mL of yeasts grown at OD₅₉₅ ~ 0.5 were pelleted 3 minutes at 4000rpm and washed in 20mL of water. Pellets were resuspended in water and washed with LiAcTE (0.1M Lithium acetate, 10mM Tris pH7.5, 1mM EDTA) pelleted and resuspended in 100µL of LiAcTE. For each transformation, 2.5µL of boiled DNA carrier at 10mg/mL and up to 15µL of DNA were added to cells and incubated 10 minutes at room temperature. After incubation, 260µL of PEG-LiAcTE (LiAcTE plus 40% PEG4000) was added before 30 minutes of incubation at room temperature. Yeasts were heat shocked 5 minutes at 42°C, pelleted and resuspended in media. The yeasts were plated on permissive media for 1 day follows by velvet replication on selective media containing antibiotics. Positive clones were patched on the selective media and certified by colony PCR before sequencing by Eurofins Genomics.

Transformation of expression plasmid with metabolic selection do not requires permissive media and can be directly plated on media lacking the metabolic component.

Yeast transformation with CRISPR-Cas9

The protocol for yeast transformation with CRISPR-Cas9 was the same that normal yeast transformation. The only difference was the composition of DNA used for the transformation. The CRISPR-Cas9 needs a special mix. The mix is composed of a plasmid encoding Cas9, sgRNA generated by Phusion PCR and repair DNA with homologous recombination arms. This method comes from (Zhang et al., 2018) and is explained in details further.

Mating

The mating of two strains were performed by mixing them on ME and leaving them mate at 25°C for 2 days at least. Then the ascus were digested with β-glucuronidase/arylsulfatase at 25°C overnight. Remaining cells were resuspended in 1mL of water with 0.5µL of 20% SDS (Final concentration of 0.1%) to lyse remaining cell membranes and cells were then grown at 32°C on adequate medium for selected desired cells. Mating type were determined with colony PCR using primers 727, 728 and 729. Mating type were also determined by using iodine assay

which coloured ascus in purple after 2 minutes exposition at iodine crystals which method is lethal for yeasts.

GST-tagged proteins purification and quantification

GST purifications were performed on Poly-Prep Chromatography Columns (#731-1550 BioRad). 400mL of cells were grown above $OD_{595} \sim 0.6$ and induced 2h with 4mL of 100mM IPTG (I6758-1G Sigma-Aldrich). After induction, cells were centrifuged at 4500rpm 4°C for 20 minutes. Decanted pellets can be stored at -20°C. Cells were resuspended in 4°C PBS and sonicated five times for 10 seconds with 2 minutes intervals. Samples were centrifuged at 8000rpm 4°C for 10 minutes twice. Supernatant were transferred on column prepared with Glutathione Sepharose 4B (17-0756-01 GE Healthcare). After three washes with 2mL of 4°C PBS, GST-tagged proteins were eluted with glutathione in four fractions of 200μL.

The fractions were prepared for electrophoresis by mixing 10μL of proteins and 10μL of 2X β-mercaptoethanol (5μL of 2-mercaptoethanol #1610710 Bio-Rad and 95μL of 2x Laemmli Sample Buffer #161-0737 Bio-Rad). Samples were loaded on a Bio-Rad mini-PROTEAN TGX gel 4-15% and run for 45 minutes at 150V. Gel was coloured with Coomassie blue (0.25g of Coomassie, 400mL of MeOH, 70mL of acetic acid for 1L) for 1h and washed with bleaching buffer (400mL of MeOH, 70mL of acetic acid and water for 1L) overnight. Gel was dried for 2h at 80°C with model 583 gel dryer (Bio-Rad).

Bradford assay was performed to quantified fractions. Standard curve was generated with BSA. OD_{595} was measured between 5 and 10 minutes after addition of 950μL of 5x Protein Assay Dye Reagent (#500-0006 Bio-Rad) to samples with spectrophotometer (Ultrospec 2100 pro Amersham Biosciences).

RNA extraction

All manipulations were performed with RNase free materials and DEPC treated water (Diethyl pyrocarbonate D5758-50ML Sigma-Aldrich).

Yeasts were grown at $OD_{595} \sim 0.5$ and pelleted after washing step with 1mL of DEPC water. Pellets which were not used directly were stored at -80°C. RNA extraction was realised with phenol-chloroform method. Pellets were resuspended in 750μL of TES (Tris EDTA SDS) and 750μL of phenol: chloroform 5:1 (P1944-400 Sigma-Aldrich) and incubated at 65°C for 1h with intervals shaking at 1300rpm. This lysis step was followed by centrifugation at 14000rpm 4°C for 15 minutes. 700μL of supernatant was retrieved and equivalent volume of phenol-chloroform –isoamyl alcohol mixture (77619-500ML Sigma-Aldrich) was added. After 5 minutes of centrifugation at 14000rpm 4°C, 650μL supernatant was retrieved and equivalent volume of chloroform –isoamyl alcohol mixture (25666-500ML Sigma-Aldrich) was added and samples were centrifuged again for 5 minutes at 14000rpm 4°C. 500μL of supernatant was retrieved and 1.5mL of -20°C ethanol was added before 50μL of 3M NaAc buffer solution (S7899-100ML Sigma-Aldrich). Samples were precipitated at -20°C overnight or at -80°C for 30 minutes. They were centrifuged 10 minutes at 4°C and washed with 70% ethanol before air drying for 10 minutes. RNA pellets were then dissolved in water and quantified with Nanodrop. RNA had been be stored at -80°C.

The RNA purification had been performed with RNeasy Mini Kit (74106 Quiagen) following manufacturer instructions.

RTqPCR

Reverse transcription had been performed with High-Capacity cDNA Reverse Transcription Kit (4368814 Applied Biosystems): 1μL of MultiScribe reverse transcriptase (50U/μL)

(4308228 Applied Biosystems) mixed with 2 μ L of 10x RT buffer, 0.8 μ L of 100mM dNTPs mix, 2 μ L of 10x RT Random primers and DEPC water for a total volume of 10 μ L per sample. This master mix was added to 10 μ L of RNA samples at 1 μ g/20 μ L. The samples were then loaded on thermal cycler programmed as explained by manufacturer.

RTqPCR had been performed with CFX96 Touch Real-Time PCR Detection System (Bio-Rad). cDNAs (diluted 100x) were mixed with adequate primers at 10 μ M and 2x Taq Universal SyberGreen SuperMix (172-5124 Bio-Rad). The PCR Detection System were programmed as follows: 3 minutes at 95 $^{\circ}$ C, 15 seconds at 95 $^{\circ}$ C and 30 seconds at 60 $^{\circ}$ C repeated 39 times.

Small RNA enrichment

All manipulations were performed with RNase free materials and DEPC treated water (Diethyl pyrocarbonate D5758-50ML Sigma-Aldrich).

Small RNAs were enriched by precipitating high molecular weight RNA with PEG. Between 200 μ g to 400 μ g of RNA were resuspended in 300 μ L of DEPC water and mixed with 40 μ L of 5M NaCl and 90 μ L of 35% PEG8000. Samples were incubated 30 minutes on ice and centrifuged at 13000rpm 4 $^{\circ}$ C for 20 minutes. 400 μ L of supernatant were restrained and mixed with 50 μ L of 3M NaAc pH5.6 and 1.5mL of -20 $^{\circ}$ C 100% EtOH. Small RNAs were precipitated at least 1h at -80 $^{\circ}$ C or overnight at -20 $^{\circ}$ C. Samples were centrifuged at 13000rpm 4 $^{\circ}$ C for 10 minutes and pellets were washed with 95% EtOH before air drying for 10 minutes and resuspension in 30 μ L. RNA pellets were then dissolved in water and quantified with Nanodrop.

RNA migration

Acrylamide gel

All used materials were treated with RNase ZAP and rinsed with DEPC water. Samples were loaded on a self-made 9.6% poly acrylamide urea gel (2.5mL of 10x TBE-Tris Borate EDTA buffer, 10.5g of urea, 6mL of acrylamide 40%, 8.2mL of DEPC water, 25 μ L of TEMED and 125 μ L of 10% APS). Gel had to be run once without samples for 30 minutes at 25mA with running buffer 1x TBE. Samples were prepared by mixing 8 μ L of small RNA (1 μ g/ μ L) to 8 μ L of 2x sample buffer (100 μ L of formamide, 1 μ L of EDTA pH8.0, 1 μ L of 2% bromophenol blue). They were denaturised 2 minutes at 95 $^{\circ}$ C and loaded on gel. Gel had been run between 30minutes to 2h at 25mA (600V) in 1x TBE and transferred on Amersham Hybond-xL membrane (RPN203S GE Healthcare) with semi-dry transfer (Owl HEP-1 ThermoFisher Scientific) for 45 minutes at 400mA (6V). The membrane was crosslinked at 80 $^{\circ}$ C for 2h.

Agarose gel

Samples were loaded on a self-made agarose gel: 100mL of 1x FA agarose (Agarose (6g/500mL), 10x FA buffer and DEPC water), 1.8mL of formaldehyde 37% and 2 μ L of Ethidium bromide (10mg/mL Sigma-Aldrich). 10x FA buffer was made with 800mL of DEPC water, MOPS 200mM, NaAc 50mM and 10mM EDTA pH 8. pH was adjusted to 7 with NaOH. Gel had to be run once without samples for approximately 10 minutes at 30V with running buffer 1x FA buffer. Samples were prepared by mixing between 10 to 30 μ g of RNA, 1.5 μ L of 10x Loading dye buffer (1mL of EDTA, 5% glycerol, 0.2% of bromophenol blue, 0.2% of xylene cyanol and DEPC water) and DEPC water to reach 15 μ L. They were denaturised 5 minutes at 65 $^{\circ}$ C and loaded on gel. Gel had been run for 2h at 100V in 1x FA buffer. Three steps of washing were realized: 15 minutes in NaOH 50mM and two times 15 minutes in Neutralization buffer (NaCl 1.5M, TrisHCl pH 7.4, 0.5M and DEPC water). Between each washing the gel was rinse with DEPC water. Samples were then transferred on Amersham Hybond-xL membrane (RPN203S GE Healthcare) by capillarity blotting overnight in 10x SSC. The membrane was crosslinked at 80 $^{\circ}$ C for 2h.

Radioactive labelling

ATP α -32P

The membranes were pre-hybridized with 9mL of PerfectHyb Plus Hybridization buffer (H7033 Sigma-Aldrich) at 42°C at least 15 minutes. The radioactive probes were made with Random Primers DNA Labelling System (18187-013, Invitrogen) by mixing 25ng of purified PCR product with 2 μ L of dATP solution, 2 μ L of dGTP solution, 2 μ L of dTTP solution, 15 μ L of random primers buffer mixture, 1 μ L of Klenow, 5 μ L of α -32P dCTP (BLU013H250UC Perkin Elmer, 10mCi/mL) and 23 μ L of DEPC water. The mix was incubated 1h at 25°C. The radioactive probe was purified with Illustra Probequant kit (GE28-9034-08 GE Healthcare) following manufacturer instructions. After denaturation step of 5 minutes at 95°C, the probe was then added to pre-hybridized membrane and incubated overnight at 42°C. Three washes at 25°C had followed the incubation: 15 minutes with 2x SSC and SDS 0.1% and two times 10 minutes with 0.1x SSC and 0.1% SDS. The membrane was dried, exposed to radiographic film and revealed with Fuji Medical Film Processor (FPM-100A Fujifilm).

ATP γ -32P

The membranes were pre-hybridized with 9mL of PerfectHyb Plus Hybridization buffer (H7033 Sigma-Aldrich) at 42°C at least 15 minutes. The radioactive probes were made by mixing 2 μ L of 5'-unphosphorylated oligonucleotides 10 μ M, 2 μ L of 10x PNK buffer, 1 μ L of T4 PNK (M410A Promega), 2 μ L of γ -32P ATP (NEG002A250UC Perkin Elmer, 10mCi/mL) and 13 μ L of DEPC water. The mix was incubated 1h at 37°C. The radioactive probe was purified with Illustra Probequant kit (GE28-9034-08 GE Healthcare) following manufacturer instructions. After denaturation step of 5 minutes at 95°C, the probe was then added to pre-hybridized membrane and incubated overnight at 42°C. Three washes at 25°C had followed the incubation: 15 minutes with 2x SSC and SDS 0.1% and two times 10 minutes with 0.1x SSC and 0.1% SDS. The membrane was dried, exposed to radiographic film and revealed with Fuji Medical Film Processor (FPM-100A Fujifilm).

Western blot

NaOH protein extraction

The extractions of proteins were realised by total alkaline extraction method. Cells were grown at OD₅₉₅ ~ 0.5 and 10 mL were centrifuged 4 minutes at 4000rpm. Pellet was washed with water and resuspended in 300 μ L of water. Then, 300 μ L of NaOH 0.6M was added before 10 minutes incubation. Cells were pellet and resuspended in 70 μ L of alkaline extraction buffer (60mM of Tris-HCl pH 6.8, 4% of β -mercaptoethanol, 4% of SDS, 0.01% of bromophenol blue, 5% of glycerol and water). Samples were denatured 10 minutes at 70°C before centrifugation.

Acrylamide gel migration

Gel migration were performed with 4-15% precast Mini-PROTEAN TGX gels (456-1083 BioRad). The gel was assembled in Mini-PROTEAN Tetra Cell (BioRad) filled with running buffer 1x. The heated protein extracts were loaded on gel. PageRuler Plus Prestrain Protein Ladder (#26619 Thermo Fischer Scientific) was used as ladder. The gel had been run approximatively 45 minutes at 150V (until migration line reach reference line).

Membrane transfer

The transfers were performed with Trans-Blot Turbo Transfer system (BioRad). The membrane used were comprised in Tran-Blot Turbo Transfer Pack (1704158 BioRad). Programs used were selected according to manufacturer recommendation.

Chemiluminescent immunostaining and revelation

Membranes were blocked with PBS 0.05% Tween 20 and skimmed milk (blocking solution) 1h at room temperature or overnight at 4°C. Once blocked, specific primary rabbit antibody diluted in blocking solution was incubated 1h at room temperature. It was followed by 3 washing step of 15 minutes each with PBS 0.05% Tween (wash solution). The secondary antirabbit antibody coupled with HRP (horseradish peroxidase) was incubated with blocking solution 1h at room temperature followed by three washing steps of 15 minutes with washing solution. A last washing with PBS was performed to rinse the membrane before revelation. The chemiluminescent reaction was performed with Western Lightning Plus-ECL kit (NEL104001EA PerkinElmer) according manufacturer protocol. The revelation of chemiluminescence was assessed with Luminescent image analyser (ImageQuant LAS 4000 GE Healthcare).

γ -toxin integration in *S. pombe* at *urg1* locus

Integration of γ -toxin-kanR gene

The pDH854 containing γ -toxin-kan sequence was generated with Phusion PCR (primers 3254 and 3255) to amplified γ -toxin from pDH722 followed by ligation in pDH34. The restriction enzymes used for the restriction were NdeI and AscI. The newly formed plasmid was transformed in DH10b cells and purified with Miniprep. γ -toxin-kanR was amplified by Expand PCR with primers 3268 and 3269. This PCR product were transformed in strains 94 and 2048 plated on EMM-N+G+kan. Primers 3270 and 3271 were used to check integration of γ -toxin-kanR gene with colony PCR. Primers 3270 and 3306 were used to check presence of *urg1* gene with colony PCR.

Integration of γ -toxin gene

Ura4 was amplified by Expand PCR with primers 3369 and 3370 from pDH364. This PCR product had been transformed in strains 96 and 674. EMM was used to check integration of *ura4* gene. The newly formed strains, 2060 and 2061 respectively, were transformed with γ -toxin amplified by Expand PCR with primers 3371 and 3372 from pDH722. EMM containing 5FOA was used to check integration of *ura4* gene.

Integration of kanR gene

KanR was amplified by Expand PCR with primers 3354 and 3355 from pDH864. This PCR product were transformed in strains 34 and 2054 on YES supplemented with kanamycin. Primers 3270 and 3356 were used to check integration of γ -toxin-kanR gene with colony PCR.

Integration of γ -toxin gene with CRISPR-Cas9

The CRISPR-Cas9 method was performed according to (Zhang et al., 2018) method. The pDH824 and pDH825 were digested with NotI. The sgRNA (A and B) were amplified by Phusion PCR with primers 3103 and 3421 (A) or 3422 (B) from digested pDH825. The DNA mix (15 μ L) for transformation was composed of 150ng of sgRNA (A), 150ng of sgRNA (B), 30ng of digested pDH824 and 1 μ g of γ -toxin amplified by Expand PCR with primers 3371 and 3372 from pDH722. This DNA mix was transformed in strains 2060 and 2061 on EMM supplemented with uracil and blasticidine. Primers 3270 and 3271 were used to check integration of γ -toxin gene with colony PCR. Primers 3270 and 741 were used to check presence of *ura4* gene with colony PCR.

Quantification of *Purg1*:: γ -toxin induction

Drop test

Drop test was performed with fresh culture of yeasts. Four dilutions were realised for each strain (10^0 , 10^1 , 10^2 and 10^3) and 5 μ L of each dilution were spotted on EMM supplemented with

uridine. EMM was also supplemented with arginine (100 μ g/mL) and/or with uracil at high (0.25 μ g/mL) or low concentration (0.072 μ g/mL). Strain 96 (WT) and 2074 (*Purg1:: γ -toxin*) were used.

RTqPCR

All cultures were grown to OD₅₉₅ ~ 0.5 at 32°C before induction. RNA was extracted as explained above after induction of *urg1* with uracil (0.25mg/mL). Expression level was measured at three timing: 0, 30 and 60 minutes after addition of uracil. RTqPCR was normalized with actin. Primers 739 and 740 were used for actin detection, primers 3305 and 3306 were used for *urg1* detection and primers 3453 and 3454 were used for γ -toxin detection.

Transformation of expression plasmids with γ -toxin under *nmt1*, *nmt41* and *nmt81* promoters

The construction of expression plasmids was performed by the integration of γ -toxin or HA-tagged γ -toxin under the control of *nmt1*, *nmt41* and *nmt81* promoters. Each plasmid was generated in the same way. The gene was amplified by Phusion PCR with primers 3361 and 3362 for γ -toxin and 3361 and 3363 for HA-tagged γ -toxin from pDH722. Restrictions were performed with BamHI and Sall restriction enzymes. After bacteria transformation and Miniprep, the genes were checked by sequencing. The newly generated plasmids are: pDH862, *Pnmt1* γ -toxin; pDH863, *Pnmt1* γ -toxin-HA; pDH864, *Pnmt41* γ -toxin; pDH865, *Pnmt41* γ -toxin-HA; pDH866, *Pnmt81* γ -toxin and pDH867, *Pnmt81* γ -toxin-HA. Each plasmid was then transformed in strains 13 and 1308 on EMM supplemented with thiamine.

Pnmt1, *Pnmt41* and *Pnmt81* induction and quantification

Drop test

Drop test was performed with fresh culture of yeasts which growth 22h or 24h without thiamine (with thiamine for controls). Four dilutions were realised for each strain (10⁰, 10¹, 10² and 10³) and 5 μ L of each dilution were spotted on EMM or EMM supplemented with thiamine. Strain 13 transformed with four expression plasmids (pDH862, pDH863, pDH864, pDH865, pDH866 and pDH867) were used. The same strain was transformed with pDH79, pDH596 and pDH621 for negative controls (the two first) and positive controls (the last one). Plates were incubated at 32°C for 24h. The first test compared pDH79 to pDH863, pDH865 and pDH867 transformed strains and the second compared pDH596 and pDH621 to pDH863, pDH865. The non-tagged γ -toxin containing plasmids were finally not used.

Zymo-HA western blot

NaOH protein extraction was performed on strain 13 transformed with the same transformed strain as drop test grown in EMM with thiamine (for controls) or in EMM without thiamine in liquid culture. Samples were then loaded on gel and revealed as explained before with chemiluminescence. The first test compared pDH79 to pDH863, pDH865 and pDH867 transformed strains and the second compared pDH596 and pDH621 to pDH863, pDH865.

Measurement of *S. pombe* resistance to *K. lactis*

Eclipse assay was performed with fresh culture of yeasts. Four dilutions were realised for each strain (10⁰, 10¹, 10² and 10³) and 5 μ L of each dilution were spotted on YES and YPD. Five strains of *S. cerevisiae* were used: 8 (*elp3::natR*); 12 (wild type); 15 (*ctk1::kanR*); 7 (*ncs6::kanR*) and 11 (wild type). Two strains of *S. pombe* were used: 94 (wild type) and 450 (*elp3::natR*). After drops were dried, *K. lactis* (IG02) was inoculated at the edge of the spots. Plates were incubated at 32°C for 24h.

γ -toxin purification for *in vitro* tests

A GST-tagged γ -toxin gene was constructed with Phusion PCR by γ -toxin amplification from pDH722 with primers 3277 and 3278. The restriction had been realised with BamHI/XhoI restriction enzymes and the ligation into pDH461 (pGEX-4T1) was performed as described previously. The newly generated plasmid, pDH855 was transformed in DH10b competent cells and diagnostic restriction was performed with NotI and PacI restriction enzymes. The construction was certified with sequencing. pDH855 was then transformed in BL21 competent *E. coli*. After heat shock, cells were incubated 30 minutes at room temperature. Plates were incubated 2 days at 25°C to slow the growth rate. GST- γ -toxin was induced with IPTG and purified as described before. The second fraction (0.83 μ g/ μ L) and third fraction (0.47 μ g/ μ L) had been conserved at -80°C until utilisation. Crude extracts were generated in the same way but manipulations were stopped before column purification.

In vitro test of GST- γ -toxin activity

The cleavage of tRNAs followed the protocol of (Lu et al., 2005). The reaction was performed at 30°C during 1h in a self-made buffer. The buffer contains 10 mM Tris-HCl, 10 mM MgCl₂, 50 mM NaCl, and 1 mM dithiothreitol (pH 7.5). The tRNAs cleaved were extracted from strains 2060 and 2061 and enriched in small RNA as explained before. Between 3 and 5 μ g of tRNAs were cleaved with different quantity of GST- γ -toxin from second fraction or crude extracts and loaded on a 9.6% poly acrylamide urea gel. The membrane obtained were radiolabelled with specific γ -32P labelled probes. These probes hybridize 5' and 3' region of tRNA^{Lys}_{UUU} (Primers 3402 and 3403) and tRNA^{Gln}_{UUG} (Primers 3438 and 3439). The radiographic films were exposed during different timing. When duration of exposition exceeds 2h, the cassette was put at -80°C to enhance the exposition.

Quantification of *urg1* induction

All cultures were grown to OD₅₉₅ ~ 0.5 at 32°C before induction with adequate component. The first experiment was dedicated to measure expression of Urg1 induced with uridine or uracil. RNA was extracted as explained above after induction of *urg1* with uracil (0.25mg/mL) or uridine (0.25mg/mL). Due to insolubility of uracil, it was needed to put uracil in media before autoclaving to dissolve it. The concentration was decided following concentration used in a publication from Carr A. (Watson et al., 2013). Induction was measured at four timing: 0, 15, 30 and 60 minutes after addition of uracil or uridine. RTqPCR was normalized with actin. Primers 739 and 740 were used for actin detection and primers 3305 and 3306 were used for *urg1* detection.

The second experiment was dedicated to assess the induction of Urg1 with lower concentration of uracil and to test impact of arginine on the induction. RNA was extracted as explained above after induction of *urg1* with uracil at 0.25mg/mL (add before autoclaving to improve solubility), uracil at 0.25mg/mL with arginine (100 μ g/mL) or uracil at 0.072mg/mL (add after autoclaving from stock solution). This concentration was determined by the highest concentration reachable in a 50x stock solution (3.6mg/mL). Above this concentration, uracil is not soluble. Induction was measured at four timings: 0, 15, 30 and 60 minutes. RTqPCR was normalized with actin. Primers 739 and 740 were used for actin detection and primers 3305 and 3306 were used for *urg1* detection.

The last experiment was a triplicate of *urg1* induction with uracil at 0.072mg/mL after 0, 15, 30 and 60 minutes. RTqPCR was performed with the same standardising gene and same primers.

The Northern blot was performed as explained before with the same RNA samples as RTqPCR. The samples induced with uridine at 0.25mg/mL, uracil at 0.25mg/mL and uracil at 0.072mg/mL were loaded on gel. The membrane was radiolabelled with α -³²P Random Primers labelling from Colony PCR product (Primers 1181 and 3306) and exposed at different timing. When duration of exposition exceeds 2h, the cassette was put at -80°C to enhance the exposition.

Appendices

Table 1. List of strains used for the project

Saccharomyces cerevisiae			
Base	Description	Genotype	Mating type
6	wt	ade2-101 ura3-52 leu2-d1 lys2-101 trp1-d63 his3-200	alpha
7	ncs6::kanR	ura3-D0 leu2-D0 his3-D1 met15-D0	a
8	elp3::natR	ura3-D0 leu2-D0 lys2-D0 his3-D1	alpha
11	wt	ura3-D0 leu2-D0 his3-D1 met15-D0	a
12	wt	ura3-D0 leu2-D0 lys2-D0 his3-D1	alpha
15	ctk1::kanR	ura3-D0 leu2-D0 lys2-D0 his3-D1	alpha

Saccharomyces pombe			
Base	Description	Genotype	Mating type
13	wt	leu1-32	h-
19	wt	ade6-216 ura4-D18 leu1-32	h+
20	wt	ade6-210 ura4-D18 leu1-32	h-
94	wt	/	h-
96	wt	ura4-D18	h-
450	elp3::natR	ade6-210 ura4-D18	h+
674	elp3::natR	ura4-D18	h-
1308	elp3::natR	leu1-32	h-
2048	elp3::ura4 (WRONG!)	ura4-D18	h-
2054	elp3::natR	/	h-
2058	urg1::kanR	/	h-
2059	elp3::natR urg1::kanR	/	h-
2060	urg1::ura4	ura4-D18	h-
2061	urg1::ura4 elp3::natR	ura4-D18	h-
2074	urg1::γ-toxin	ura4-D18	h-

Table 2. List of plasmids used for the project

Base	Vetor	Selection marker	Gene
pDH34	pFA6A	AmpR-KanR	TAP
pDH79	pREP3	LEU2-AmpR	/
pDH94	pREP41	LEU2-AmpR	/
pDH269	pREP81	LEU2-AmpR	/
pDH364	pKS	ura4-AmpR	ura4
pDH461	pGEX-4T1	AmpR	CTD S2A
pDH596	pREP41	LEU2-AmpR	Isk1 F353G
pDH621	pREP41	LEU2-AmpR	Isk1-HA (m7SA p5SE)
pDH722	pGEX4-T1	AmpR	GST-mug187 cDNA
pDH824	pDB4281	AmpR	Cas9 gapped plasmid / split bsdMX
pDH825	pDB4283	AmpR	Cas9 gapped insert / split bsdMX
pDH854	pFA6a	AmpR-KanR	γ-toxin (Zymocin)
pDH855	pGEX-4T1	AmpR	γ-toxin (Zymocin)
pDH862	pREP3	LEU2-AmpR	γ-toxin
pDH863	pREP3	LEU2-AmpR	γ-toxin
pDH864	pREP41	LEU2-AmpR	γ-toxin
pDH865	pREP41	LEU2-AmpR	γ-toxin
pDH866	pREP81	LEU2-AmpR	γ-toxin
pDH867	pREP81	LEU2-AmpR	γ-toxin

Table 3. Sequences of primers used for the project

Base	Full name	Sequence (5' to 3')
720	nmt F	GGTTCAGTCACCCAACG
727	MT1	AGAAGAGAGAGTAGTTGAAG
728	MP	ACGGTAGTCATCGGTCTTCC
729	MM	TACGTTCACTAGACGTAGTG
739	*act1 +948 F	CCACTATGTATCCCGATTGTC
740	*act1 +948 R	CAATCTTGACCTTCATGGAGCT
741	ura4 R3	AGAGAAGCTGGTTGGAAGGC
1306	nmt1 term R	GTACTCGTTGTCGGAGATCAAG
3103	split-bsdMX R	GGCCGCATCTTCACTGGTGTC
3254	Ndel-zymocin F	ATGGAATTccatgATGgcagctactactgcg
3255	zymocin-Ascl R	ggcgccttatacacattttccattctgtagatttc
3268	Zymo-KanR F	ccattgaacatcgttgtcattttctgtgttttactatccgttttctcaactactaaatagaactaattcaatATGGCAGCTACTACT GCGAG
3269	Zymo-KanR R	AATGAAGGTAATTAACACATGTATGTGAAATTTAAAAATAACATGGTCCTTCTGTGACGTCTAAAAACAGATG GGCAAGCgaattcgagctcgtttaaa
3270	Zymo-KanR check F	TAAGGGAGGAAATCCATACGG
3271	Zymo-KanR check R	aatttcatctctctaccaagacc
3277	BamHI-GST-Zymo F	CGGGATCCgagctactactgagag
3278	GST-Zymo-Xhol R	cgggcCTCGAGttatacacattttccattctgtagatttc
3305	Urg1 F	GAAGCATGATGCCATTGTTGG
3306	Urg1 R	CCTTCTCAGAGAAGTAACCG
3354	urg1::KanR F	ttcgtttgtcattttctgtgttttactatccgttttctcaactactaaatagaactaattcaatAGATCTGTTAGCTGCTCG
3355	urg1::KanR R	AATGAAGGTAATTAACACATGTATGTGAAATTTAAAAATAACATGGTCCTTCTGTGACGTCTAAAAACAGATG GGCAAGCgtttaaactggatggcg
3356	urg1::KanR Check R	CGAGACGAAATACGCGATCG
3361	Sall-Zymo F	acccgaGTCGACatggcagctactactgagag
3362	Zymo-BamHI R	gcgGGATCCtatacacattttccattctgtagatttc
3363	Zymo-HA-BamHI R	gcgGGATCCtaAGCATAATCTGGAACATCATATGGATAAGCGTAATCTGGAACATCGTATGGGTAtacacatttc cattctgtagatttc
3369	urg1::ura4 F	tcgtttgtcattttctgtgttttactatccgttttctcaactactaaatagaactaattcaatCGCCAGGGTTTTCCAGTC
3370	urg1::ura4 R	AATGAAGGTAATTAACACATGTATGTGAAATTTAAAAATAACATGGTCCTTCTGTGACGTCTAAAAACAGATG GGCAAGCgccaagctcgaattaaccc
3371	urg1::zymo F	tcgtttgtcattttctgtgttttactatccgttttctcaactactaaatagaactaattcaatATGGCAGCTACTACTGCG
3372	urg1::zymo R	AATGAAGGTAATTAACACATGTATGTGAAATTTAAAAATAACATGGTCCTTCTGTGACGTCTAAAAACAGATG GGCAAGCtatacacattttccattctgtagatttc
3373	zymo F (sequençage)	GCTCTATAAAAGACGGTGACT
3402	5'probe lysine tRNA	GCCGATTGAGCTAAACGGGA
3403	3'probe lysine tRNA	CTCCCACTGCGAGACTCGAA
3404	5'probe arginine tRNA	aGCCATTAGGCCACGGGAGC
3405	3'probe arginine tRNA	AGCTCCCGCGGGACTCGAA
3406	5'probe glycine tRNA	TTGATGTACCGCTACCCA
3407	3'probe glycine tRNA	TGCATCGACCGGGAGTCGAA
3408	5'probe serine tRNA	aACCACTCAGGCATAGTGC
3409	3'probe serine tRNA	GCAGGATTTGAACCTGCGCG
3421	sgRNA ura4a	ATAGTTGCTGTTGCCAAAAACATAACCTGTACCGAAGAA-TTTTATCTTGTGTCTACA- GTTTTAGAGCTAGAAATAGCAAG
3422	sgRNA ura4b	ATAGTTGCTGTTGCCAAAAACATAACCTGTACCGAAGAA-AAATGCATACATATAGCCAG- GTTTTAGAGCTAGAAATAGCAAG
3438	5'probe glutamate tRNA	aGCCGTTGGACCACAACGGA
3439	3'probe glutamate tRNA	CTCCGTTGCGGGAGTCGAA
3453	Zymo +404 F	AGAAAACGGAGAACCCGAC
3454	Zymo +404 R	CCTTATAACCTCAACACTATTAGAG

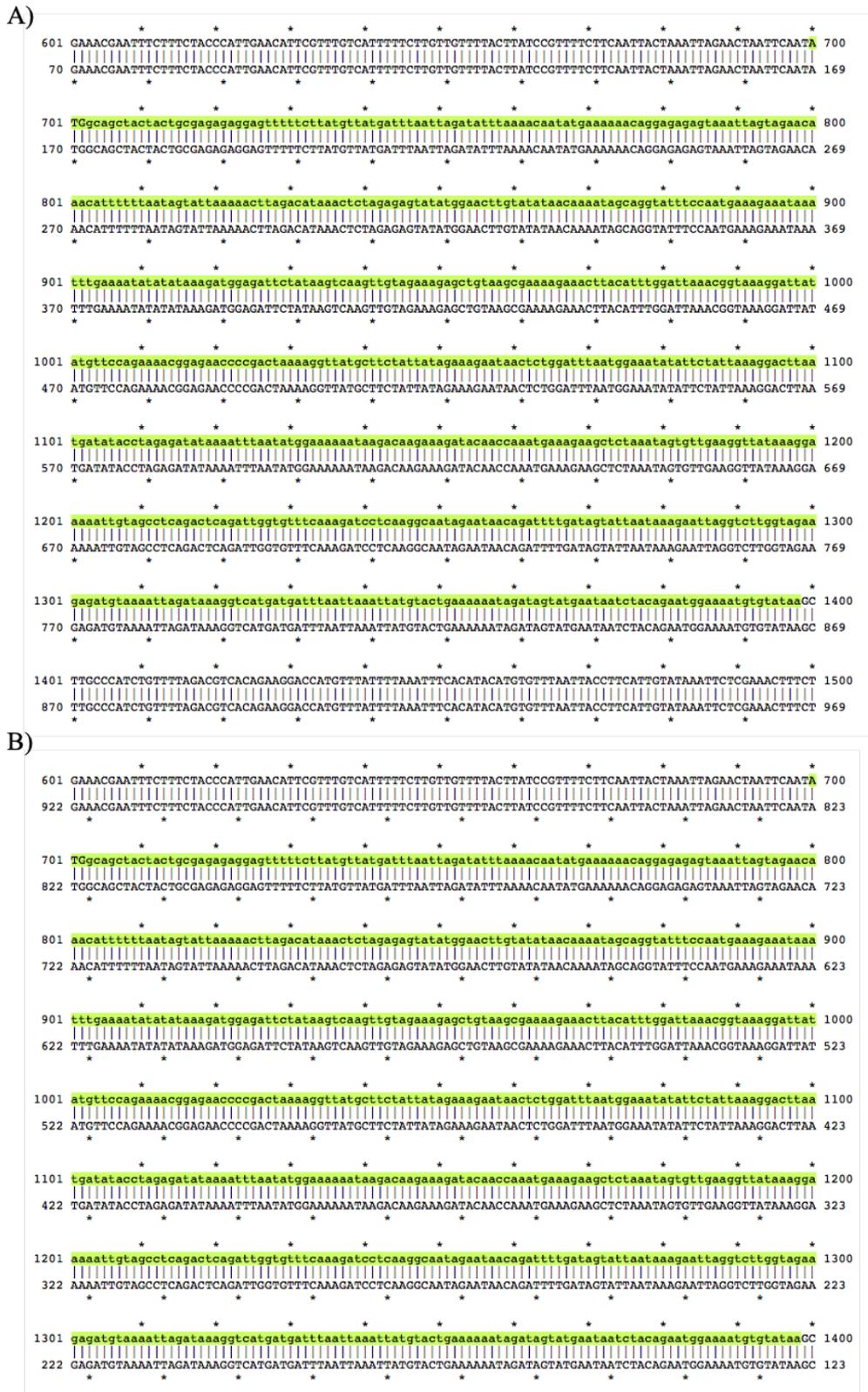


Figure S1. Sequencing of γ -toxin at *urg1* locus. A) Forward sequencing. B) Reverse sequencing

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