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WADJET in a eukaryotic system

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Abstract

The discovery of CRISPR has shown how valuable research into bacterial immune systems can be. Since then, many new and diverse defense systems have been unearthed in the bacterial pan-genome. Particularly unique and promising among these is the plasmid defense system WADJET. It consists of four proteins, three of which form an SMC complex sensing the topology of the DNA substrate, likely by loop extrusion. The remaining fourth component is a nuclease responsible for cleavage. Together they selectively recognize and cleave circular DNA up to a certain size limit. In this thesis we aimed to test WADJET for the first time in a eukaryote, the budding yeast *S. cerevisiae*. We found that the Kleisin component JetA was undetectable in its native form but succeeded in its stable expression after fusing the fluorescent protein tag Venus to its C-terminus. Expressing some or all components of the WADJET complex did not adversely affect growth of yeast. Proteins were abundantly detected in yeast nuclei. We established 2 independent assays for detecting potential plasmid loss as a result of WADJET activity, an ADE2 based color sectoring assay and a physical assay for linearization based on plasmid enrichment. We also tried to test it on chromatin free plasmids using transformation efficiency as a proxy. However, without further modifications, expression of the WADJET complex did not alter plasmid stability and did not cause any linearization. Our assay for transformation with naked DNA proved non informative, because, unexpectedly, our induction system using estrogen and the transcription activator Gal4.ER strongly affected plasmid stability, transformation efficiency and cell growth.

1. Introduction

1.1. Plasmids and horizontal gene transfer

The term plasmid, first coined by Joshua Lederberg in 1952, describes DNA elements, often circular, that can replicate independently from the chromosome.^{1,2} Plasmids exist in all domains of life; however, they are much more abundant in bacteria and archaea, with around 43% of known prokaryotic genome sequences having at least one plasmid.³ An example for a rare eukaryotic plasmid is the 2 μ plasmid found in yeast, which is a selfish genetic element with almost chromosome like stability.⁴ The reasons why plasmids occur so infrequently, even in single celled eukaryotes, compared to prokaryotes are still being investigated. One probable reason is that for bacteria, plasmids are much more important to achieve genetic diversity in a short time, whereas eukaryotes have other options like sexual reproduction. Another factor could be that uptake and transfer of plasmids is more efficient for the simpler prokaryotic cell lacking a nucleus. Nonetheless, plasmids have been adopted and widely used as cloning vectors in molecular biology for a variety of model organisms.²

Plasmids show a remarkable diversity in both structure, size, mode of transfer as well as in the traits they encode for. Some of these, like antibiotic resistance or biosynthetic pathways, are beneficial to the plasmid host. However, plasmids also have a role as selfish genetic elements, that do not provide any benefit but in fact are detrimental due to the metabolic burden incurred to the host by replicating them or are even actively harmful.^{1,2,5} This means bacteria must maintain a balance between taking up and defending against incoming foreign DNA. There are three canonical, well studied pathways for bacteria to take up and pass on plasmids, a process which is called horizontal gene transfer. Conjugation is the most common mechanism for plasmid mobility and involves direct contact between bacterial cells and the formation of a so-called conjugation pilus, through which the plasmid is transferred. Another of these canonical pathways is transformation, where free DNA is taken up directly from the environment. This is expected to occur rather

infrequently in nature, but it is however the predominant mechanism by which plasmids are introduced into a bacterial cell in a laboratory setting. Lastly there is also plasmid uptake by transduction, which happens when bacteria are afflicted by a bacteriophage that can transfer a plasmid payload.^{1,6}

1.2. Bacteriophages

Bacteriophages are a type of virus that infect prokaryotes. They are the single most abundant biological entity on earth. At least in marine environments, there are an estimated 10 phages for every bacterial cell.⁷ Naturally they are also associated with human microbiota and have since the 1910s been used as therapeutic agents, due to their ability to kill bacteria without harming humans. After falling out of favour due to the advent of antibiotics, they are still being used and are experiencing a resurgence as therapeutics.⁷⁻⁹ This is owed to their specificity and ability to kill antibiotic resistant pathogens.

There are two different types of life cycle bacteriophages can adopt the lytic and lysogenic cycle. In the lytic cycle, after adsorption to the target cell and delivering their DNA, the host replication machinery is hijacked to multiply the viral DNA.^{7,8} Phage particles are produced, and the cell is lysed, releasing the packaged phages. During the lysogenic cycle, the phage DNA replicates passively together with the chromosome and can either integrate into it or exist as a plasmid.¹ Under certain conditions the phage can switch to the lytic cycle. In this lysogenic state, the phage can even increase the hosts fitness by providing, for example, resistance to infection of other phages or virulence factors.^{1,6-8} In any case the ubiquity of phages and the complex interplay with their hosts have caused an evolutionary arms race between bacteria and their viruses, with huge selective pressure applied to systems that convey resistance to phages specifically and foreign DNA more generally.¹⁰

1.3. Bacterial defense against phages and plasmids

To cope with the constant exposure to foreign DNA, either plasmid or phage, with a variety of beneficial and harmful effects, bacteria have developed defence mechanisms not unlike the eukaryotic immune system.¹⁰⁻¹⁴ They can be adaptive or more indiscriminate. Some of these systems, like restriction enzymes and CRISPR/CAS have changed the landscape of biological science.¹⁵ Fortunately, these systems tend to be clustered within the prokaryotic genome in so called “defence islands”. This makes it possible to more easily identify such potential defence systems with a genomics approach. Only in recent years has there been an explosion of newly discovered bacterial defence systems with huge mechanistic diversity and untold potential for research.¹⁰

Regarding phages, several systems have been identified, targeting different stages of the phage infection. The first line of defence is to prevent the adsorption of the phage to the cell. Bacteria can achieve this by a plethora of ways, like biofilm formation or masking surface receptors. Once the phage DNA is injected, bacteria still have several options of counteracting the infection. Some undergo programmed cell death in an altruistic response to prevent the spread of the phage, while others inhibit virus particle packaging.⁸ However historically, the most impactful systems are those that attack the phage DNA directly.

Restriction enzymes have been discovered in the 1950s and have since been an invaluable tool in molecular biology. They have evolved as bacterial defence mechanism against phage and plasmid DNA and occur in 80-90% of known bacterial genomes.¹⁶ They recognize specific DNA motifs, usually 4-8 bp, and modification patterns to discriminate between self and nonself. Incoming DNA usually lacks the methylation pattern created by the bacterial methyltransferases and is cleaved. These restriction enzymes have been characterized into four categories based on structure and restriction behaviour, with type II being the most extensively studied and used. This is due to them introducing site specific cuts in or close to their recognition sequence with either blunt ends or overhangs. Naturally, plasmids and phages have co-evolved with these restriction-modification

systems and have developed strategies to evade them. They achieve this either by losing or occluding the restriction sites, mimicking methylation or actively inhibiting the enzymes themselves.^{8,17}

A slightly different type of defence systems uses CRISPR/Cas. Much like the restriction system, CRISPR/Cas cleaves DNA, but uses a guide RNA that is complementary to the target for recognition. This guide RNA is incorporated into the CRISPR locus via a prior encounter with the phage or plasmid, making the CRISPR system adaptive and heritable. For Type-II CRISPR/Cas systems, like the commonly used Cas9, the active complex consists of a trans-activating RNA (tracrRNA) that binds to the repeats interspaced between the guide RNAs (spacers) forming an RNA duplex. This then associates with the Cas proteins to form the active complex. The CRISPR/Cas system has proven to be a precious tool for genetic engineering and is regarded as one of the most important scientific discoveries in the last decades. It allows researchers to make precise and efficient changes in the genome of many different organisms. The system could readily be adapted for use in eukaryotes, with modifications being adding a eukaryotic promoter, codon optimization, adding 5' and 3' UTR sequences to allow for efficient expression of the relevant genes as well as expressing the guide RNA from a non-coding RNA promoter (e.g. pSNR52) and nuclear localization signals (NLS) on Cas9. Many variants of the system exist, using different Cas proteins with varying effector domains, which allow not only for cleavage but also for instance transcriptional activation of the target.¹⁵

The immense success of the CRISPR/Cas system has increased interest in other bacterial defence systems. A landmark study by Doron and colleagues in 2018 has revealed many new systems that show anti phage or plasmid activity. Among these are some that confer resistance to plasmids and contain proteins that belong to the “structural maintenance of chromosomes” or SMC class.¹⁰

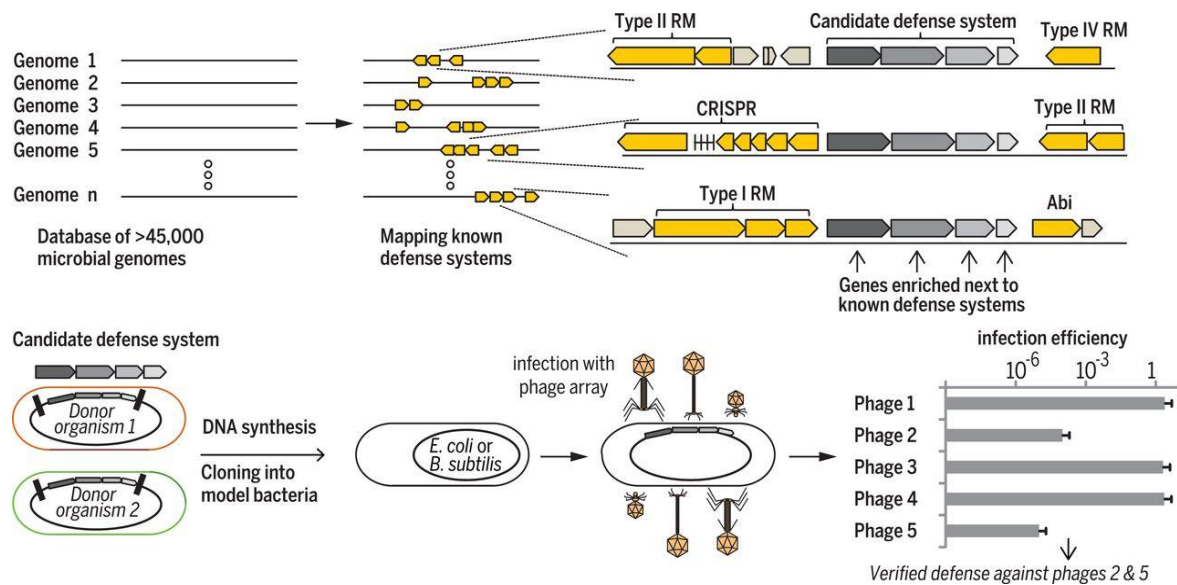


Figure 1 Scheme for the systematic discovery of phage/plasmid defence system in the bacterial pan genome from Doron et al. 2018

1.4. SMC proteins

SMC proteins occur in all domains of life and are structurally conserved across them. They perform many different tasks like chromosome compaction, organization and defence against invading DNA. They usually consist of three subunits, the SMC motor which has a hinge domain, for dimerization with another motor protein, as well as a head domain with ATPase activity and a coiled-coil domain involved in creating a cavity for DNA capture. There are also the kleisin subunit which in turn recruits either KITE (kleisin interacting winged helix tandem elements) or HAWK (HEAT repeat proteins associated with kleisins) proteins allowing for DNA entrapment, the latter having only been found in eukaryotes.^{18,19} One promising model for how SMC complexes work is through loop extrusion. By translocating DNA through their ringlike structure, distant domains can be brought into proximity, facilitating chromosome organization. This process requires ATP hydrolysis, but the exact mechanism is still being investigated, with different models for

different structures of SMC complex having been proposed. One key feature in these models is the topological entrapment of DNA in a ringlike structure formed by the components of the SMC complex.^{18,20}

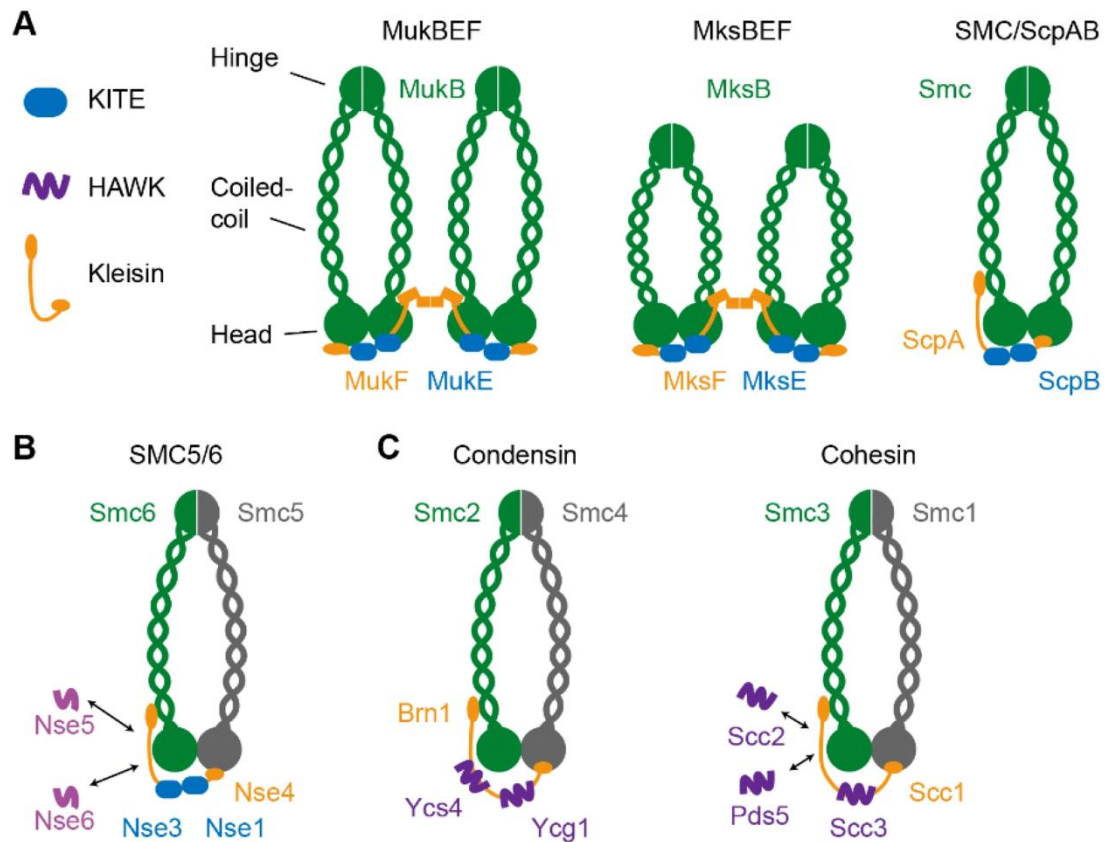


Figure 2 (A) Structure of assorted bacterial SMC proteins (B) Structure of SMC5/6 (C) Structure of Cohesin/Condensin, adapted from Mäkele and Sherrat 2025

In eukaryotes, three different SMC complexes are present, condensin, cohesin and SMC 5/6. They promote chromosome compaction, sister chromatid cohesion and DNA repair. Prokaryotes invariably possess at least one essential SMC complex.^{18,20} Examples of conventional bacterial SMCs include MukBEF from *E.coli* and SMC ScpAB from *B.subtilis*, which have roles in sister chromatid separation and segregation.¹⁹ In contrast to their eukaryotic counterparts, prokaryotic SMCs often form homodimers. Recently SMC 5/6 was reported to be somehow involved in reducing viral infection²¹, and also dedicated defence systems containing SMC components have been discovered.¹³

1.5. Loop Extrusion

Since 1882, it has been known that DNA can form loop structures.¹⁸ Until the 1990s the prevailing view has been that these loops are anchored to a protein scaffold.^{20,22} An alternative model more in line with experimental observations was proposed by Arthur Riggs.^{20,23,24} He suggested that proteins could reel in DNA, forming loops, and thereby establish physical proximity of elements that are far apart on a sequence level. This hypothesis has since been further developed with many findings supporting it.²³ However, the exact molecular mechanism of loop extrusion has remained elusive.

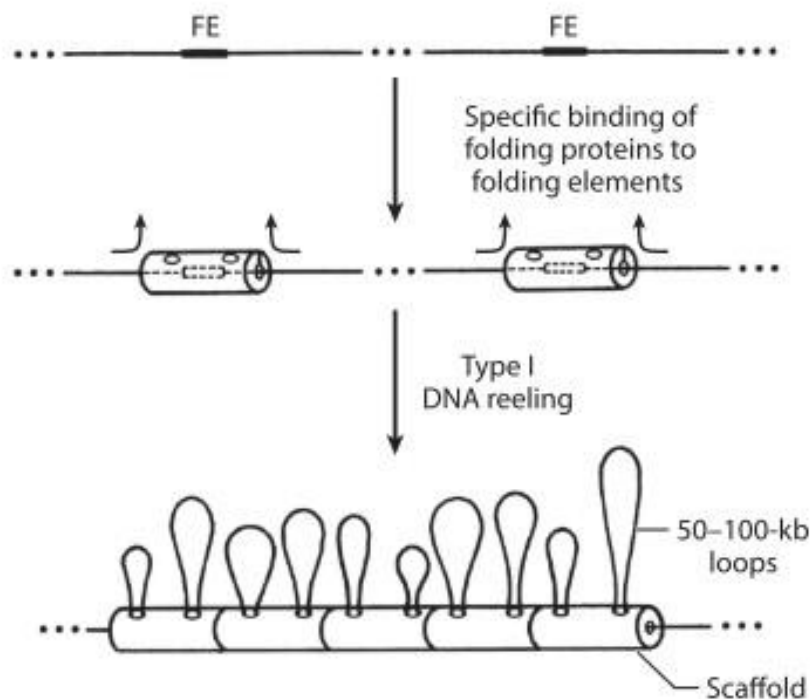


Figure 3 Loop extrusion model as proposed by Riggs.²⁴Adapted from reference 18/48. FE = Folding Element.

1.6. WADJET

One defence system that uses a particularly unique way of recognizing foreign DNA is called WADJET/JetABCD. Aptly named after a god protector of ancient Egypt, it is present in around 5-10% of bacterial genomes and can selectively sense and cut circular DNA up to a size limit of 100 kb.^{10,14} WADJET-type systems were first discovered through mutations in *Mycobacterium smegmatis* that increased the efficiency of plasmid transformation (former name of WADJET=efficiency of plasmid transformation) before being unearthed in many different bacterial genomes after Doron and colleagues 2018 study.¹⁰ Three different types of WADJET-systems have been identified, of which type I is the focus of this thesis. In contrast to other well established defence systems, like CRISPR, sequence does not matter for WADJET, only circularity and size do.²⁵

WADJET consists of four components, namely JetA,B,C and D. JetABC form an SMC like complex that was shown to be structurally similar to the known bacterial SMC protein MukBEF, while JetD has a TOPRIM domain related to the TopoVI/Spo11-transesterase family.²⁵ JetABC are analogous to kleisin, KITE and SMC motor respectively, found in canonical SMC complexes. All four proteins are required for DNA cleavage in an ATP dependant manner.¹⁴ The proposed mechanism is that JetABC form a complex capable of loop extrusion, with one functional unit consisting of 2xJetC 2xJetB 1xJetA, that again forms a dimer with another motor unit via interaction of their JetA subunits.^{14,19,25,26} JetA and JetB together also form a compartment for DNA entrapment. The two connected dimers then extrude DNA symmetrically.²⁷ Current evidence suggests that JetD, which occurs as a homodimer, is not a stable part of the complex but rather is recruited to the DNA after loop extrusion by JetABC inevitably stalls when circular DNA is extruded. This model is supported by recent data that directly show WADJET's capability for loop extrusion in vitro as well as completion of loop extrusion being required for cleavage.²⁷ Additionally, it has been shown that it is indeed circularity that is required for recognition and cleavage of DNA, not just the absence of free ends.²⁸

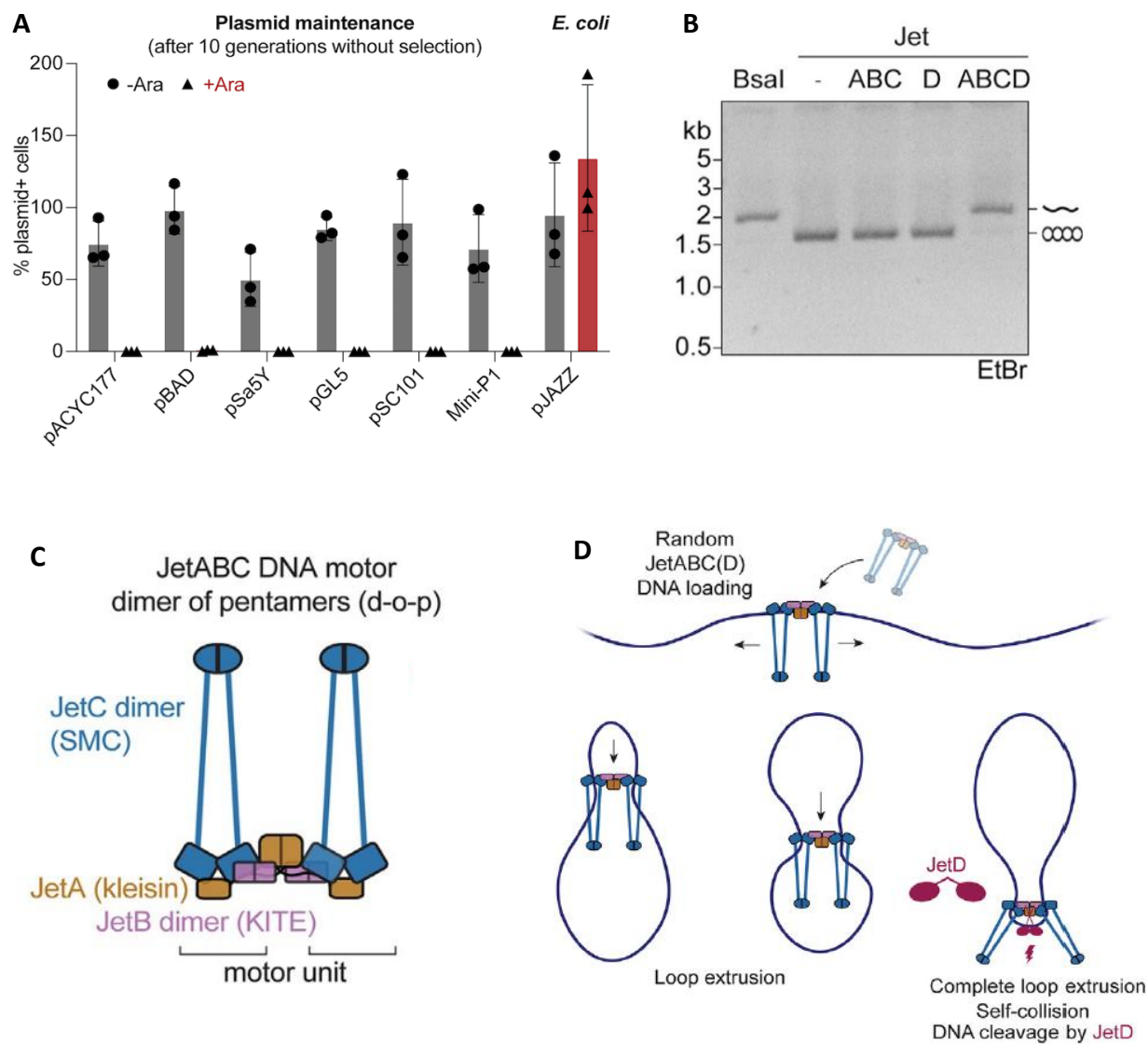


Figure 4 (A) Effect of the induction of the WADJET system on the maintenance of several plasmids (pJAZZ is linear) in *E. coli* (B) Cleavage assay with and without individual WADJET components (C) Cartoon of JetABC structure (D) Scheme for the proposed mechanism of WADJET plasmid recognition and cleavage adapted from Rosiné-Hameling, Gruber and colleagues 2022/2024

Recent findings of the Gruber lab also give insight into the mode of DNA binding and entrapment. Their Cryo-EM data suggest a DNA boarding state that is initiated by ATP binding, where a neck gate between JetC and JetA is opened and closed by rotation of JetC upon ATP hydrolysis. This happens sequentially on the two different functional units resulting in a half loaded intermediate state and finally a loaded state where both complexes face in opposite direction. Upon completion of loop extrusion, the exact mechanism of which is still unclear, WADJET undergoes further conformational changes finally resulting in a cleavage competent state that recruits JetD.²⁶

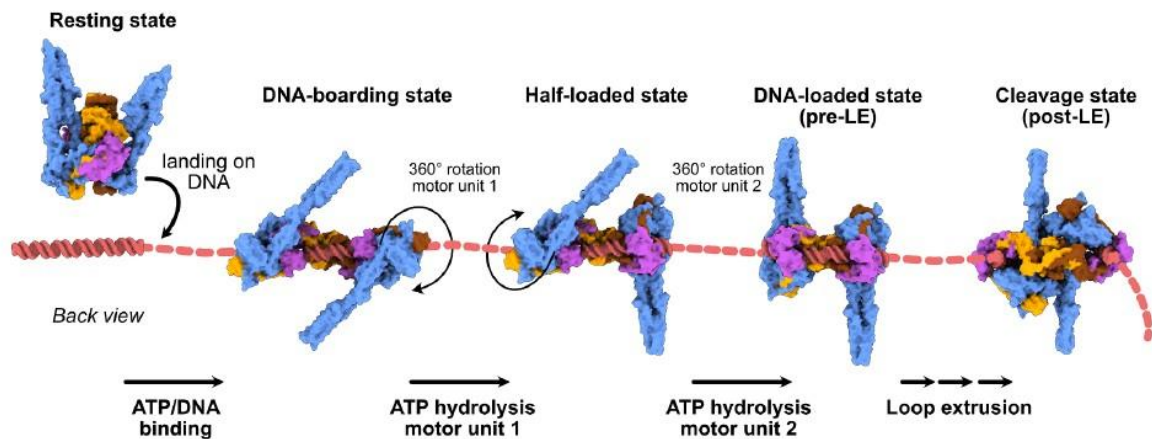


Figure 5 Backside overview of known WADJET Type I states during loading/loop extrusion, adapted from Rosiné-Hamelin, Gruber et al. 2025

WADJET has proven valuable as a model to study loop extrusion and through its ability to selectively recognize and cleave circular DNA, it may offer applications if it were able to recognize circular DNA in Eukaryotes as well. This could position it as an anti-viral agent independent of viral sequence. Therefore, it would be advantageous to characterize WADJET in a eukaryotic system.

1.7. Aims of this thesis

History has shown that investigating bacterial immune systems can yield immense theoretical and practical benefits for science and technology. Studying the WADJET system in a eukaryote could address whether the proteins can be stably expressed, whether it shows any activity *in vivo*, causes DNA damage or interferes with chromosome dynamics or whether it attacks for instance mitochondrial DNA.

The yeast *Saccharomyces cerevisiae* is a well-established, simple eukaryotic model system and therefore represents an ideal starting point to address these questions regarding WADJET in eukaryotes.

The goal of this work was to express a Type I WADJET system from *E. coli*, kindly provided by Stefan Gruber, in *Saccharomyces cerevisiae* strain SK1. We aimed to study and find conditions for the expression of the Jet proteins to test its ability to cleave chromatinized and naked plasmids. We employ a widely used colour assay, where the loss of an ADE2 gene containing plasmid results in a switch from white to red colonies. Finally, cleavage, if occurring, should be observed directly via Southern Blot.

2. Materials and Methods

2.1. Media and solutions

2.1.1. Liquid media yeast

2.1.1.1. YPD+

10 g Yeast Extract (Difco, #212750)
20 g Peptone (Difco, #211677)
20 g D-(+)-Glucose monohydrate (Sigma-Aldrich, #16301-1KG)
250 mg Ade/Ura/Trp mix (5 ng each)
added dH₂O to 1 L
sterilized using 0.2 µm MF75 Bottle-Top-Filter PES (Thermo Fisher, #3470570)

2.1.1.2. SPS medium

1.7 g Yeast Nitrogen Base w/o amino acids and
 w/o ammonium sulfate (Difco, #233520)
10 g Ammonium Sulfate
5 g Yeast Extract (Difco, #21²⁷50)
10 g Peptone (Difco, #211677)
10 g Potassium Acetate (Sigma-Aldrich, #P1190-1KG)
10.2 g Potassium hydrogen phthalate (Merck, #1.04874.1000)
added dH₂O to 1 L
adjusted to pH 5.5 using 10 M KOH, sterilized using 0.2 µm MF75 Bottle-Top-Filter PES
(Thermo Fisher, #3470570), stored at 4 °C

2.1.1.3. SPM medium

20 g Potassium Acetate (Sigma-Aldrich, #P1190-1KG)
added dH₂O to 1 L
autoclaved at 121 °C for 20-30 min, stored at room temperature (RT)

2 mL of following amino acid mix were added to 1 L SPM:

Amino acid (AA) mix for SPM for meiotic time course (all AAs from Sigma-Aldrich)

100 mg	Uracil
100 mg	Tryptophan
100 mg	Histidine
100 mg	Arginine
150 mg	Leucine
added dH ₂ O to 50 mL	

sterilized using a 0.2 µm syringe filter, stored as 2 mL aliquots at -20 °C

2.1.1.4. Synthetic Minimal -ADE/-URA/-TRP

For dropout, omit the appropriate ingredient.

1.7 g	Yeast Nitrogen Base w/o amino acids and w/o ammonium sulfate (Difco, #233520)
5 g	Ammonium Sulfate
11 g	Casamino acids (Difco, #223120)
55 mg	Adenine (omit for -ADE)
55 mg	Tyrosine
55 mg	Uracil (omit for -URA)
20 g	Glucose

added dH₂O to 990 mL

autoclaved at 121 °C, 20-30 min, then added:

After cooling down to about 55-60 °C, added:

5 mL	Leucine (10 g/L, sterilized using 0.2 µm syringe filter)
5 mL	Tryptophan (10 g/L sterilized using 0.2 µm syringe filter) (omit for -TRP)

Or weigh in and dissolve everything and sterilize with 0.2 µm syringe filter.

2.1.1.5. Synthetic Minimal –ARG/-HIS/-LEU/-LYS

1.7 g Yeast Nitrogen Base w/o amino acids
and w/o ammonium sulfate (Difco, #233520)

5 g Ammonium Sulfate

55 mg Adenine (Omit for -ADE)

55 mg Tyrosine

55 mg Uracil (Omit for -URA)

20g Glucose

added dH₂O to 990 mL

autoclaved at 121 °C, 20-30 min, then

After cooling down to about 55-60 °C, added:

10 mL 100x Amino acid drop out mix

Or weigh in and dissolve everything and sterilize with 0.2 µm syringe filter

100x Amino acid drop out mix (omit respective amino acid for dropout)

400 mg L-Lysine (4 mg/L) (omit for -LYS)

800 mg L-Leucine (8 mg/L) (omit for -LEU)

300 mg L-Isoleucine (3 mg/L)

200 mg L-Methionine (2 mg/L)

2000 mg L-Threonine (20 mg/L)

600 mg L-Tryptophan (6 mg/L)

500 mg L-Phenylalanine (5 mg/L)

200 mg L-Histidine (2 mg/L) (omit for -HIS)

200 mg L-Arginine (2 mg/L) (omit for -ARG)

4000 mg L-Serine (40 mg/L)

1400 mg L-Valine (14 mg/L)

added dH₂O to 100 mL

and sterilized using 0.2 µm syringe filter

2.1.2. Solid media yeast

2.1.2.1. YPD+/YPD plates (and G418/ClonNat/Hygromycin B)

10 g Yeast Extract (Difco, #212750)
20 g Peptone (Difco, #211677)
250 mg Ade/Ura/Trp mix (5 ng each) omit for YPD
20 g Agar (LabM, #MC002-A)

added dH₂O to 900 mL

autoclaved at 121 °C, 20-30 min

After autoclaving, added:

100 mL Glucose (200 g/L, sterilized using 0.2 µm MF75
 Bottle-Top-Filter PES (Thermo Fisher, #3470570))

For G418/ClonNat/Hygromycin B plates – Antibiotic medium

After cooling down to about 55-60 °C, added 1 mL antibiotic stock solution:

200 mg/L G-418 Disulphate (ForMedium, #G4185)
100 mg/L ClonNat (Werner BioAgents, #5001000)
300 mg/L Hygromycin B (ForMedium, #HYG5000)

2.1.2.2. SPM plates

10 g Potassium Acetate

added dH₂O to 1 L

20 g Agar (LabM, #MC002-A)

autoclaved at 121 °C for 20-30 min

2.1.2.3. Synthetic Minimal plates -ADE/-URA/-TRP

1.7 g Yeast Nitrogen Base w/o amino acids
and w/o ammonium sulfate (Difco, #233520)

5 g Ammonium Sulfate

added dH₂O to 900 mL and adjusted pH 5.5-6.0 using KOH

20 g Agar (LabM, #MC002-A)

autoclaved at 121 °C, 20-30 min

After autoclaving, added:

100 mL Glucose (200 g/L, sterilized using 0.2 µm MF75 Bottle-Top-Filter
PES (Thermo Fisher, #3470570))

SD-ADE/-URA/-TRP plates - synthetic drop out media

For dropout, omit the appropriate ingredient.

1.7 g Yeast Nitrogen Base w/o amino acids
and w/o ammonium sulfate (Difco, #233520)

5 g Ammonium Sulfate

11 g Casamino acids (Difco, #223120)

55 mg Adenine (omit for -ADE)

55 mg Tyrosine

55 mg Uracil (omit for -URA)

added dH₂O to 900 mL, adjusted to pH 6

20 g Agar (LabM, #MC002-A)

autoclaved at 121 °C, 20-30 min, then added:

100 mL Glucose (200 g/L, sterilized using 0.2 µm MF75
Bottle-Top-Filter PES (Thermo Fisher, #3470570))

After cooling down to about 55-60 °C, added:

5 mL Leucine (10 g/L, sterilized using 0.2 µm syringe filter)

5 mL Tryptophan (10 g/L sterilized using 0.2 µm syringe filter)
(omit for -TRP)

2.1.2.4. Synthetic Minimal plates -ARG/-HIS/-LEU/-LYS

1.7 g Yeast Nitrogen Base w/o amino acids
and w/o ammonium sulfate (Difco, #233520)

5 g Ammonium Sulfate

55 mg Adenine

55 mg Tyrosine

55 mg Uracil

added dH₂O to 890 mL, adjusted to pH 6

20 g Agar (LabM, #MC002-A)

autoclaved at 121 °C, 20-30 min, then added:

100 mL Glucose (200 g/L, sterilized using 0.2 µm MF75

Bottle-Top-Filter PES (Thermo Fisher, #3470570))

After cooling down to about 55-60 °C, added:

10 mL 100x Amino acid drop out mix

100x Amino acid drop out mix (omit respective amino acid for dropout)

400 mg L-Lysine (4 mg/L) (omit for -LYS)

800 mg L-Leucine (8 mg/L) (omit for -LEU)

300 mg L-Isoleucine (3 mg/L)

200 mg L-Methionine (2 mg/L)

2000 mg L-Threonine (20 mg/L)

600 mg L-Tryptophan (6 mg/L)

500 mg L-Phenylalanine (5 mg/L)

200 mg L-Histidine (2 mg/L) (omit for -HIS)

200 mg L-Arginine (2 mg/L) (omit for -ARG)

4000 mg L-Serine (40 mg/L)

1400 mg L-Valine (14 mg/L)

added dH₂O to 100 mL

and sterilized using 0.2 µm syringe filter

2.1.3. Liquid/solid media bacteria

2.1.3.1. LB

50 g Tryptone
25 g Yeast extract
50 g NaCl
20 g Agar agar (LabM, #MC002-1KG) - omit for LB liquid medium
added dH₂O to 5 L
adjusted to pH 7.4, autoclaved, stored on RT

2.1.3.2. 2x TY

80 g Tryptone/Peptone (Carl Roth, #91079-40-2)
50 g Yeast extract
25 g NaCl
20 g Agar agar (LabM, #MC002-1KG) - omit for 2x TY liquid medium
added dH₂O to 5 L
adjusted to pH 7.2, autoclaved, stored on RT

For resistance selection, the respective amounts of antibiotic stock solution were added to cooled media after autoclaving:

Antibiotic/Stock/final concentration

Ampicillin (Amp, in dH ₂ O)	100 mg/mL	100 µg/mL
Kanamycin (Kan, in dH ₂ O)	50 mg/mL	30-50 µg/mL
Chloramphenicol (CAM, in EtOH)	34 mg/mL	34-50 µg/mL

filter sterilized using a 0.2 µm syringe filter and aliquots were stored at -20 °C.

2.1.3.3. SOC

20 g	Peptone (Carl Roth, #8986.4)
5 g	Yeast extract (Difco, #212750)
584 mg	Sodium chloride (Sigma Aldrich, #71382-1KG)
625 mg	Potassium chloride (Carl Roth, #6781.1)
2.03 g	Magnesium chloride hexahydrate (Applichem, # A1447.1000)
2.46 g	Magnesium sulphate heptahydrate (Sigma Aldrich, #63140-1KG-F)
3.96 g	D-(+)-Glucose monohydrate (Sigma-Aldrich, #16301-1KG)

added dH₂O to 1 L

Adjusted to pH 7.4 using NaOH, autoclaved

2.1.3.4. SOB

20 g	Peptone (Carl Roth, #8986.4)
5 g	Yeast extract (Difco, #212750)
584 mg	Sodium chloride (Sigma Aldrich, #71382-1KG)
625 mg	Potassium chloride (Roth, #6781.1)

added dH₂O to 1 L

Adjusted to pH 7.4 using NaOH, autoclaved

2.2. Bacterial Methods

2.2.1. Gibson Assembly

Gibson Assembly was performed according to D.G. Gibson (Gibson, 2011).

5x ISO reaction buffer

750 mg (250 g/L)	PEG-8000
1.5 mL (500 mM)	Tris-HCl pH 7.5, 1 M
150.0 µL (50 mM)	MgCl ₂ , 1 M
300.0 µL (50 mM)	DTT, 0.5 M
30.0 µL (1 mM)	dGTP, 100 mM
30.0 µL (1 mM)	dATP, 100 mM
30.0 µL (1 mM)	dTTP, 100 mM
30.0 µL (1 mM)	dCTP, 100 mM
10.0 mg (5 mM)	NAD

added dH₂O to 3 mL

aliquoted and stored at -20 °C

Gibson master mix (Enzyme-reagent master mixture)

100 µL	5x ISO reaction buffer
0.20 µL	T5 Exonuclease (10 U/µL) (NEB, #M0363)/
6.25 µL	Phusion® High-Fidelity DNA Polymerase (2 U/µL) (NEB, #M0530)
50 µL	Taq DNA ligase (40 U/µL) (NEB, #M0208)
218.55 µL	dH ₂ O (added to final Volume of 375 µL)

Or without Ligase

100 µL	5x ISO reaction buffer
0.20 µL	T5 Exonuclease (10 U/µL) (NEB, #M0363)/
6.25 µL	Phusion® High-Fidelity DNA Polymerase (2 U/µL) (NEB, #M0530)
268.55 µL	dH ₂ O (added to final Volume of 375 µL)

aliquoted to 15 µL and stored at -20 °C

The DNA fragments to be assembled were mixed in a volume not exceeding 5 µL, calculations were done based on 50-100ng of backbone with 2-10x molar excess for

the fragments depending on size. Very short fragments were joined via bridge PCR. Assembly reactions were incubated for 1-2h at 50°C and 2-10 µL were transformed into DH5α.

2.2.2. Preparation of competent *E. coli* DH5α

1. Inoculate a DH5alpha *E. coli* single colony into 25 mL of SOB medium in a 50 mL Erlenmeyer flask. Incubate with shaking at 37°C overnight.
2. Inoculation of Main Culture: Transfer 0.5 mL of the fresh overnight culture into 50 mL of fresh SOB medium ($OD_{600} = \text{ca } 0.05$) in a 200 mL Erlenmeyer flask. Incubate (shaking) at 30°C until OD_{600} reaches 0.35. This should take approximately 4 hours.
3. 10' Cooling: Transfer the culture into pre-chilled 50ml Falcon tubes and place on ice.
4. 12' Pelleting Bacteria: Centrifuge at 4000 rpm ($4.239 \times g$) @ 4°C, 12 minutes in a cooled benchtop centrifuge. Discard the supernatant carefully by decanting slowly without disturbing the soft pellet.
5. 10' Resuspension in Inoue Transformation Buffer: Gently but thoroughly resuspend the pellet in 20 mL of ice-cold Transformation Buffer until no particles are visible.
6. 10' cooling on Ice: Place the suspension on ice for 10 minutes.
7. 12' Second Centrifugation: Repeat step 4.
8. 10' Final Resuspension: Gently but thoroughly resuspend the pellet in 3 mL of ice-cold Inoue Transformation Buffer plus 225 µL DMSO.
9. 10' Final Incubation on Ice: Place the suspension on ice for another 10 minutes.
10. Aliquoting and Freezing: Aliquot 50 µL of the suspension, freeze in liquid nitrogen.

Inoue Transformation buffer:

Reagent	Quantity (for 1 L)	Final concentration
MnCl ₂ ·4H ₂ O	10.88 g	55 mM
CaCl ₂ ·2H ₂ O	2.20 g	15 mM
KCl	18.65 g	250 mM
PIPES (0.5 M, pH 6.7)	20 mL	10 mM
H ₂ O	800 mL	

PIPES has to be added shortly before use it is stored frozen, put @ R.T. 1-2hours before use!

2.2.3. Mini-and Midi Prep

For mini prep Promega Wizard® Plus SV Minipreps DNA Purification Systems (A1330) was used according to manufacturer's specification. Usually, two columns were eluted in the same volume of water to increase concentration. Midi prep was performed using Promega PureYield™ Plasmid Midiprep (A2492) according to manufacturer's instructions.

2.2.4. T4 Ligation

Ligation was performed using NEB T4 DNA ligase (M0202) following the manufacturer's protocol.

2.2.5. Transformation of *E.coli* DH5α

Aliquot of competent cells was left to thaw on ice for approximately 10 min. Afterwards DNA to be transformed was added (usually 2 µL Gibson Assembly Mix or 1 ng plasmid DNA) and cells were left to incubate on ice for at least 20 min. Subsequently heat shock was performed at 42°C for 60 sec and afterwards 950µL of SOC were added. The cells were put back on ice for 5 minutes after which they were allowed to recover for 45 min at 37°C. Then the volume was reduced by centrifugation and the cells pelleted was resuspended in 150µL of LB or SOC. The resuspended cells are plated on 2xTY or LB plates with appropriate antibiotic added.

2.3. Yeast Methods

2.3.1. Yeast Transformation

Yeast o/n culture was inoculated from plate, alternatively cells can be diluted in a manner that would result in desired cell density ($OD_{600}=0.4-0.8$) at a certain timepoint. Usually strains to be transformed are grown in YPD+, however transformation can also be performed when inoculating in selective media. When starting from stationary culture, 50 ml of medium are inoculated using this o/n culture to $OD_{600}=0.1-0.2$. Desired cell density should be reached after approximately 4:30h.

Afterwards the culture is harvested by centrifugation at 3000 rpm for 5 min. Resulting cell pellets are washed in 1 mL 0.1 M LiAc by resuspending, transferring to 2 mL Eppendorf vial and spinning down again at 3000 RPM for 5 min. The washed cells are then resuspended in 150-300 μ L 1 M LiAc (depending on cell density).

Each transformation contains:

8 μ L sheared salmon sperm DNA (boiled for 10 min beforehand)

2-15 μ L DNA (250-500ng plasmid, 750-1000ng linearized integration plasmid, 3-6 μ g PCR product)

90 μ L 50 % PEG 3350 ssDNA

24 μ L cells in 1 M LiAc

Once cells in LiAc are added, the suspension should only be mixed by gently flipping. The transformation mix is incubated for 45 min at RT, after which 12 μ L 60 % glycerol are added. After another 45 min at RT, heat shock is performed for 10 min at 42 °C. When plating on auxotrophic markers, the volume of transformation mix is brought up to 200 μ L with water and then plated.

When selecting for antibiotic resistance (e.g. KanMX, NatMX, HphMX), cells were incubated at RT for at least 4 h or o/n after adding 1 mL YPD+ before plating, ensuring sufficient oxygen in the vial, usually done by making a hole in the lid with a canula. Before plating, volume was reduced by centrifugation and in case of o/n incubation, cells can be divided between two plates.

2.3.2. Yeast Plasmid Isolation

Plasmid DNA was isolated from Yeast using the Zymoresearch Zymoprep Yeast Plasmid Miniprep II (D2004) according to manufacturer's protocol. When recovering plasmid for southern blot, the protocol for exponential liquid culture was used.

2.3.3. Yeast Cross

Strains to be crossed of complementary mating type were mixed on YPD+ plate using an autoclaved toothpick and incubated at 30°C o/n for subsequent sporulation and dissection or 4h for zygote isolation.

2.3.4. Zygote isolation

Zygotes were identified by their propeller-like shape and separated using a Leitz Micromanipulator. The isolated zygotes were then left to grow on YPD+ at 30°C for further use.

2.3.5. Sporulation and dissection

Mating mix is spread onto SPM plate and left to incubate at 30°C overnight when working with pure SK1 or for up to 3 days at RT when working with BY derived strains.

Successful sporulation was verified under microscope. Roughly a 200 µL pipette tip of the sporulation patch was resuspended in 192 µL dH₂O. 3 µL Zymolyase (10 mg/ml) and 5 µL 0.5 M DTT were added and left to incubate at 30 °C for 20 min or 30 min at RT.

After the digest, 20 µL of the suspension were applied to top center of a YPD⁺ plate and the plate was tilted to create a uniform line of spores in the middle of the plate. The cell suspension was left to dry for at least 15min. Tetrads were dissected using the Leitz micromanipulator equipped with a glass needle.

The plate was incubated at 30 °C for 2 days or until colonies appear. Genotype of spores was determined by replica plating on respective markers or if necessary, colony PCR.

2.3.6. In situ immunostaining of yeast

Solutions:

DAPI 0.2g/ml in water, store at 4°C in dark

DTT dithiothreitol (0.5M in water, store at -20°C)

ZYMOLYASE T100 10mg/ml, in water, store at -70°C

poly-L-Lysine 0.1% in water

Blocking buffer 1%BSA, 1% Gelatine in PBS

PBS 1x

Preparing slides and fixing the samples:

Drops of poly-L-Lysine were added to the wells of a 30-well microscopy slide. After 5 min in a metal container containing damp paper towels for moisture the drops were aspirated off. Wells are washed with 10 µL of water. Dried slides can be frozen for later use.

Fix and digest:

To 1 ml sample culture, 100 µl formaldehyde are added. Samples can be stored o/n or up to 7 days at 4°C. Samples are spun 4 min at 4500 rpm (4°C) and pellets are washed 3x with 1 mL 4°C TBS.

Resuspend the pellet in:

20 µL 2M Sorbitol

150 µL DMEM cell culture medium + 1M Sorbitol

30 µL 0.5 M DTT

10 µL Zymolase (T100, 10 mg/mL)

(add just ½ or ¾ of all components if pellets are small)

The suspension is incubated for 15-20 min at 37°C, the digest is monitored by microscopy and is stopped by placing the cells on ice. The cells can be stored long term at -20°C.

Immuno staining:

10 µl spheroblasted cells are dropped into the wells, slides are put into moisture chamber, given 10 min to settle down and liquid is gently aspirated off. Afterwards the slide is plunged into pre-chilled methanol for 3 min and acetone for 10 seconds at -20°C. After acetone has evaporated 10 µl blocking buffer are added to each well.

After 20-30 minutes of incubation the blocking buffer is removed and 10 µl of primary antibody are added. Slides are incubated for 2h at RT or o/n 4°C in a moisture chamber. After incubation, antibody solution is removed, and wells are washed in 1xPBS for 5-10 minutes. Next 10 µl of secondary antibody are added and the slide is again incubated for 2h at RT in a moisture chamber. Antibody is again removed and the slides washed in 1xPBS for 5-10 minutes. The slides are flicked dry and a drop of Vecta shield/DAPI (0.2 mg/mL) is added.

2.3.7. Yeast recombineering

In vivo assembly of DNA fragments with overlapping homologies can be achieved using *S. cerevisiae* innate ability for homologous recombination. 300ng of vector backbone were used with fragments in 2-10x molar excess depending on size. DNA was co-transformed into yeast using the method described in 2.3.1. Transformants were verified by colony PCR and plasmid isolated using Zymoresearch Zymoprep Yeast Plasmid Miniprep II (D2004).

2.3.8. WADJET plasmid stability assay

Test strains are grown in SM -HIS-ADE/-ADE at 30°C, to select for JetA-Venus 2micron plasmid and ADE2 CEN test plasmid, until OD600=0.3-0.8 (or stationary) as measured on the Denovix-DS11 photometer is reached. At this point ~300 cells (assuming OD600=1=1x10⁷ cells/mL) are plated on YPD (no additional adenine) with and without 1 µM estrogen to induce JetA/JetD. Before plating cells already appropriately diluted are sonicated 5x1sec at 25% intensity to break up aggregates. After ~5 days, red and sectorized colonies should appear, indicating plasmid loss.

2.3.9. WADJET plasmid transformation efficiency assay

Test strains are grown in SM -HIS, selecting for JetA-Venus and transformed with a CEN test plasmid following the protocol in 2.3.1. One hour before harvest cultures were split and 1 mM estrogen was added to one half. Cells were plated selecting for test plasmid and JetA-Venus with and without 1 µM estrogen added to plates. However, this method turned out to be unsuitable since the presence of estrogen in a GAL4-ER strain without any WADJET proteins already almost completely abolished transformation efficiency.

2.4. DNA Methods

2.4.1. Q5 PCR

Reaction		Program	
5x Q5 Buffer	10.0 μ l	98 °C	30 ''
2 mM dNTPs	5.0 μ l	98 °C	10 ''
Plasmid/Genomic DNA*	2.5 μ l/ 1 μ l	Ta***	30 ''
Fw Primer 10 pmol/ μ l**	2.5 μ l	72 °C	30 '' /kb
Rv Primer 10 pmol/ μ l**	2.5 μ l	72 °C	2 '
Q5 DNA Polymerase	0.5 μ l		
<u>PCR H₂O</u>		<u>27 μl/28.5 μl</u>	
Total	50.0 μ l		

35x

* **Plasmid** (approx. 25 ng), 1:100 diluted from stock; **Genomic DNA** 1 μ l

** 1:10 dilution of stock (100 pmol/ μ l) in dH₂O

*** calculated using [NEB Tm calculator](#)

2.4.2. Phire Green PCR

Reaction		Program	
2x Phire Green MM	15.0 μ l	98 °C	5 '
Fw Primer (10 pmol/ μ l)	1.5 μ l	98 °C	7 ''
Rv Primer (10 pmol/ μ l)	1.5 μ l	Ta***	7 ''
Sample**	1.5 μ l	72 °C	15 '' /kb
<u>dH₂O</u>	<u>10.5 μl</u>	72 °C	1'
Total	30.0 μ l		

40x

* 1:10 dilution of stock (100 pmol/ μ l) in dH₂O

** colony resuspended (Yeast or *E. coli*) in 20 μ l dH₂O or 10ng of DNA

*** calculated using [Thermo Scientific Tm calculator](#)

2.4.3. Bridged PCR

Very short fragments (<400bp) for DNA cloning were joined using bridged PCR. This is a two step process, in the first steps the individual fragments with overlaps are created in Q5 PCR. In the the second step these fragments are used as templates with both primer pairs, with the inner primers being diluted 1:100 with respect to outward primers.

2.4.4. Agarose gel electrophoresis

50 x TAE

242 g	Tris base
57.1 mL	Acetic Acid
18.6 g	EDTA

were added to 900 mL dH₂O

dH₂O was added to 1 L, the pH was adjusted to 7.9

Unless otherwise specified agarose-DNA gels were cast and run with 1xTAE buffer. Agarose concentration was usually between 0.8-2%, depending on fragment size. Midori green was used as a dye unless promoting the supercoiled plasmid species was desired, in this case EtBr was used.

2.4.5. Purification of DNA from gel and from PCR reactions

Purification of DNA from PCR reactions was carried out using Monarch[®] PCR & DNA Cleanup Kit (NEB, #T1030L), according to manufacturer's instructions.

Purification of DNA from agarose gels was carried out using Monarch[®] DNA Gel Extraction Kit (NEB, #T1020L), according to manufacturer's instructions.

2.4.6. DNA Precipitation

1/10 of total volume NaOAc 3M, pH 5 is added as well as 2 volumes 96% EtOH. After mixing the DNA preparation is incubated at -20°C for at least 30 minutes. Afterwards the mixture is centrifuged at full speed/4°C for 15 minutes. The supernatant is removed and 1 mL of cold 70% EtOH is added to the pellet without resuspending it. After a second centrifugation step at full speed/4°C for 10 minutes, the supernatant is again removed, and the pellet is left to dry. Once the ethanol has fully evaporated, the pellet is resuspended in dH₂O.

2.4.7. qPCR

qPCR was performed using Luna® Universal qPCR Master Mix (NEB, M3003), Eppendorf twin.tec® 96 real-time PCR Plate (Eppendorf, #30132513) on an Eppendorf Realplex Mastercycler. In brief, 20 µl reactions (18 µl MM + 2 µl sample) were set up in duplicates using filter pipette tips. Plasmid standards for quantification were used in dilutions 1E-4, 1E-6, 1E-8, 1E-10 µM. addition of samples, plates were sealed (Microseal® 'B' PCR Plate Sealing Film from BioRad, #MSB1001), vortexed shortly and centrifuged for 1 min at 3,000 rpm.

PCR mix per sample:

10 µl Luna MM

0.5 µl Primer oligo mix (10 µM)

7.5 µl ddH₂O

2 µl DNA sample

20 µl total reaction volume

Program:

95 °C, 60 sec

(95 °C, 15 sec + 60 °C, 30 sec + plate read) x 40 cycles

60-95 °C, 20 min melting curve

= roughly 1.5 h

2.4.8. Sequencing

Sanger sequencing of Plasmids and PCR products was done via the service provided by Microsynth Austria, sample preparation was as specified on [Microynth Sample Requirements](#). Full plasmid sequencing was done via Plasmidsaurus, sample requirements see [Plasmidsaurus Sample requirements](#).

2.4.9. Non-radioactive Southern Blotting for plasmid cleavage

2.4.9.1. Sampling

Strains are grown in medium selecting for relevant plasmids until OD₆₀₀=0.2-0.86, at this point WADJET is induced with 2 mM estrogen. 1.5 mL samples are removed at desired time points, and plasmid is isolated using the protocol for liquid culture provided by the manufacturer. It is important to note that higher centrifugation speeds may be required for proper pelleting of the cells. Zymolase incubation time was 45 min with 2 mM estrogen added to the digest. Final elution volume was 12 µl of dH₂O.

Final DNA yield was determined by qPCR and Qubit measurement. Yields were usually around 10 ng for endogenous 2mic plasmid and 30-40ng for 2micron and p1138 (2mic origin, 9.3kb) combined. qPCR and Qubit measurements were in good agreement.

2.4.9.2. Gel electrophoresis

5-15 ng of sample DNA were loaded per lane along with untreated and in vitro digested plasmids as controls on a 0.6% agarose gel with EtBr in 1x TAE buffer. The gel was run for 3h at 60V until linearized and circular forms were well separated. Controls for p1138 and endogenous 2µ were linearized by NheI and NdeI respectively.

2.4.9.3. Vacuum Transfer

1 L 0.25 M HCl

H ₂ O	900 ml
37 % HCl 12.178 M	20.5 ml
H ₂ O	ad 1 L

2 L Alkaline Transfer buffer

NaOH 39.99 g/mol	40 g (0.5 M)
NaCl 58.44 g/mol	175.32 g (1.5 M)
H ₂ O	ad 2 L

4 L 2x SSPE

20x SSPE (SigmaAldrich, S2015-1L)	400 ml
H ₂ O	ad 4 L

Following electrophoresis, the gel is washed 1x in 0.25M HCl for 20 min, 2x in H₂O for 5 min and 1x Alkaline Transfer buffer 5-10 min. After pre-wetting the membrane (Hybond-N+, Amersham biosciences) in alkaline transfer buffer for 60 seconds, the VacuGene XL Blotting system (Amersham BioSciences) was assembled according to manufacturer's instructions. DNA was transferred for 1.5h at 50 mbar, taking care to always keep the gel submerged by adding additional transfer buffer. Afterwards the apparatus is disassembled and the gel lifted carefully using its original tray to avoid bending and associated leakage of DNA onto the membrane. The membrane was rinsed 2x with 2xSSPE and excess liquid was blotted off by placing the membrane on filter paper. Next the membrane was crosslinked using a UV Stratalinker twice, turning the membrane 180 degrees between cycles. Subsequently the membrane was air dried before proceeding with the next steps.

2.4.9.4. Pre-hybridization

Pre-hybridization solution

2x SSPE

1 % SDS

0.5 % dry milk

10 mg fish DNA (#11467140001, Roche; boiled for 10 min just before use)

Hybridization steps were performed in glass tubes inside a hybridization oven. Depending on tube size, 5-25ml, at least enough to submerge the membrane of respective solution must be used. The membrane is rolled up lengthwise for it to fit inside the tube without overlapping, DNA side facing inwards.

The membrane is incubated in pre-hybridization solution at hybridization temperature for 1h. Hybridization temperature is calculated using the formula by Roche from their Easy HYB buffer manual. As a caveat, their buffer composition is proprietary and might not resemble the one we used, nonetheless it was chosen as a guiding point.

$$T = (49.82 + 0.41 (\%G + C) - (600/l)) - 20$$

l = length of hybrid in base pairs

2.4.9.5. DIG probe synthesis

Probes were chosen to identify all plasmids with a 2micron origin or p1138 specifically. Primers 6583 and 6592 produce probe P1(511 bp, p1138 specific). Primers 6580 and 6581 produce probe P2 (510 bp, P1138 and 2μ).

Probes were synthesized using the HighFidelity Digoxigenin PCR Labeling Kit (Jena Bioscience, APP-101-DIGX-S

Component	Volume	Final concentration
PCR-grade water	10 μl	
High Fidelity Buffer (10x)	2 μl	1x
1 mM dATP/dCTP/ dGTP working solution	2 μl	100 μM
1 mM dTTP working solution	1.3 μl	65 μM
1 mM DIG-11-dUTP	0.7 μl	35 μM
forward primer (10 μM)	1 μl	0.1 - 1 μM (e.g. 0.3 μM 500 bp forward primer)
reverse primer (10 μM)	1 μl	0.1 - 1 μM (e.g. 0.3 μM 500 bp reverse primer)
template DNA	1 μl	1 - 10 ng genomic DNA (e.g. 1 ng Lambda DNA)
High Fidelity Pol (2.5 units/μl)	1 μl	2.5 units
Total volume	20 μl	

Recommended cycling conditions

Cycle step	Temperature	Time	Cycles
Initial denaturation	95°C	2 min	1x
Denaturation	95°C	20 sec	30x
Annealing	TM°C*	30 sec	
Elongation	68°C**	60 sec	
Final Elongation	68°C	2 min	1x

*Taq calculator by Thermo Scientific

**per 500bp, 72°C also works

Successful labeling and yield is verified by loading the reaction on a gel next to a trial reaction without DIG-11-dUTP, only dTTP. Labeled probe should run at higher molecular weight. Typical yield is around 1µg. PCR probe reaction can be used directly.

2.4.9.6. Hybridization

Hybridization solution

2.5x SSPE

1 % SDS

5 % dextran sulfate 500 kDa (D8906, Sigma Aldrich)

0.5% dry milk

10 mg Salmon Sperm DNA (sheared)

Dextran sulfate will need some time to dissolve. Prepare well in advance, just after starting the pre-hybridization and keep at hybridization Temperature, so it's ready to be mixed with the labelled probe. If not pre-sheared, salmon sperm DNA is extruded through a syringe with a bent canula several times to shear.

10 µL DIG probe (~500ng) were added to 1mL salmon sperm DNA (10mg/mL), boiled for 10 minutes to denature and added to 25 mL of hybridization solution pre warmed to hybridization temperature (20ng probe/mL). Pre-hybridization solution is removed from tube, replaced with the hybridization mixture and incubated o/n at hybridization temperature (50°C). Probe-mixtures can be reused after boiling again for 10 minutes.

2.4.9.7. Membrane Washes

Washing is done inside a plastic or glass container on a rocking platform.

Washes:

2xSSPE 0.5% SDS 30°C 5 min (prewarm)

2xSSPE 0.5% SDS 25-28°C 20 min

0.2x SSPE 0.5% SDS RT 30 min

0.2x SSPE 0.5% SDS RT 10 min

0.2x SSPE 0.5% SDS 55-65°C 30 min (prewarmed)

0.2x SSPE 5 min

2.4.9.8. CSPD detection

Detection is achieved using the DIG-Luminescence detection Kit (Roche, 11363514910). All buffers and solutions are prepared as specified in the protocol. Imaging is performed on a ChemiDoc MP system (Biorad). An exposure time of 10 minutes was sufficient.

2.4.9.9. Stripping and re-probing

Stripping in 0.4M NaOH at 45°C for 30 minutes was not efficient. Harsher conditions like boiling SDS night yield better results. This is likely due to the used DIG probes not being alkali labile.

2.5. Protein Methods

2.5.1. TCA protein extract preparation

50 ml 3x GSD buffer:

187.5 mM Tris-HCl (pH 6.8)

6% (w/v) SDS

30% glycerol

150 mM DTT

0.2% (w/v) bromophenol blue

2% β -mercaptoethanol (v/v)

Filter through 0.45 μ m filter, store as aliquots on -20 °C

2.5.1.1. Sampling

For WADJET expression studies, samples were taken when cultures were in exponential phase, OD600 = 0.3-0.8. All steps were performed on ice. At the desired timepoints, a volume of culture corresponding to 6×10^7 cells (assuming OD600 = 1.0 = 1.5×10^7 cells/ml) was harvested (3000 rpm, 5 min, 4 °C, in 15 mL Falcon Tubes) and washed once with water before resuspending the cell pellet in 2 mL 20% cold TCA. Samples can be stored like this at 4 °C at least overnight for further processing. Once all samples have been collected, they are again centrifugated (3000 rpm, 5 min, 4 °C), resuspended in 1 mL of 10% cold TCA and transferred to 1.5 mL Eppendorf tubes. Supernatant is removed by centrifugation (7000 rpm, 5 min, 4°C) and pellets can be processed further immediately or frozen at -20°C for a few days or flash frozen in liquid N₂ and stored long term at -80°C.

2.5.1.2. Extract preparation

All steps are performed on ice. 200 μ L of cold 10% TCA are added to the pellets along with 200 μ L of glass beads (measured out using a 200 μ L PCR tube). The cells are then lysed by shaking on a Vortex Genie, with appropriate attachment, at full speed/4°C for 30 minutes. Using 1000 μ L pipette tips, as much as possible (around 150 μ L) of the lysate is transferred to a fresh 1.5 mL Eppendorf tube and the beads are washed twice with 200 μ L 10% TCA. The extract and the two washes are pooled and centrifugated (7000 rpm, 5 min, 4°C). The supernatant is removed,

and the pellets are resuspended in 80 μL 3x GSD + 40 μL 1M Tris-HCL pH 8.0 + 120 μL H₂O by vortexing for 5 minutes (RT). Cell extracts should retain blue color, if not the pH is too low likely due to remaining TCA and can be adjusted with Tris-HCL. Samples are boiled for 10 min at 95°C for 10 minutes and then centrifugated for 5 minutes/13000 RPM at RT. Samples can now be stored at -20°C but should be boiled for 5 minutes and centrifugated again before use in SDS-PAGE.

2.5.2. SDS-PAGE

10x SDS Running Buffer

250 mM Tris

1.89 M Glycine

1 % (w/v) SDS

10 ml Seperating Gel 10%

H₂O 3.5 mL

40 % Bis-Acrylamide 2.5 mL

1M Tris (pH 8.8) 3.8 mL

10 % SDS 100 μL

10 % APS 100 μL

TEMED 4 μL

3 ml Stacking Gel

H₂O 2 mL

40 % Bis-Acrylamide 380 μL

1M Tris (pH 6.8) 570 μL

10 % SDS 30 μL

10 % APS 30 μL

TEMED 3 μL

The gel apparatus (Mini-Protean II Cell Gel System or Mini-Protean Tetra Cell (both BioRad)) was assembled and filled completely with 1x SDS Running buffer. 8-15 μ L of sample were loaded and electrophoresis was performed at 200 V until the dye front reached the very bottom of the gel.

2.5.3. Western blot

2.5.3.1. Blotting

5x Tris/Glycine

125 mM Tris

960 mM Glycine

pH 8.0

Transferbuffer

480 ml dH₂O

160 ml 96 % Ethanol

160 ml 5x Tris Glycine

precool to 4 °C

Amersham Hybond™, 0.45 PVDF (GE Healthcare via Fisher Scientific, #15259894)

The membrane was activated in MeOH for 20 seconds, alternatively it can also be activated for 2-5 minutes in EtOH with no loss in transfer efficiency. The transfer sandwich (BioRad) was assembled as follows:

- Layer 1: 3mm foam sponge wet in transfer buffer
- Layer 2: 2 sheets of Whatman paper (10x7cm) wet in transfer buffer.
- Layer 3: Protein Gel, equilibrated in transfer buffer beforehand
- Layer 4: Activated Membrane, wetted in transfer buffer (9,5x6,5cm). Any air bubbles are removed using a 50 mL Falcon tube.
- Layer 5: 2 sheets of Whatman paper (10x7cm) wetted in transfer buffer.

- Layer 6: 2 sheets of Whatman paper (10x7cm) wet in transfer buffer. Any air bubbles are removed using a 50 mL Falcon tube.

The cassette containing the transfer sandwich is placed into the tank along with a cool pack and a magnetic stirrer. The tank is filled with transfer buffer, and the proteins are blotted at 4°C for 1-2h at 100V or o/n at 25V. When blotting at 100V, the cool pack is refreshed after half time the transfer time. The success of the transfer is checked by Ponceau S staining. Briefly, membrane is submerged in 0.1% Ponceau S solution and agitated for 5 min in plastic tray. Afterwards it is de-stained with water until good separation of the protein bands from the background is achieved. Before proceeding with the protocol, it is more thoroughly destained in water for 5-10 minutes with agitation.

2.5.3.2. Antibody Staining

1xPBST (1x PBS + 0.1 % Tween 20)

137 mM NaCl

2.7 mM KCl

4.3 mM Na₂HPO₄

1.47 mM KH₂PO₄

pH 7.4 [NaOH]

0.1 % Tween 20 (Sigma, #P1379)

Blocking solution

5 % non-fat dry milk in 1x PBST

After verifying successful transfer, the membrane is blocked in blocking solution for 1h at RT or o/n at 4°C. Next membrane is incubated with primary antibody appropriately diluted in 0.5% milk in PBST. Incubation time can be 2h at RT or o/n at 4°C. Following incubation membrane is washed 3x10 minutes in PBST and incubated with secondary antibody appropriately diluted in 0.5% milk in PBST for 1-2h at RT. Membrane is again washed 3x10 min in PBST. If HRP-conjugated antibody was used, 2 mL of mixed Clarity Western ECL substrate solution (Biorad #1705060) are evenly spread on the membrane inside a development folder. Imaging was done on a Biorad ChemiDoc MP imaging system and subsequent analysis using ImageLab software.

3. Results

3.1. Building a system for WADJET expression and analysis in Yeast

The first step in this project was to construct a system for expression and detection of all WADJET components in yeast, along with a way to screen for plasmid loss. Concerning the expression, JetABC and JetD should be expressed from separate constructs to assay the effect of both components of the system alone. Additionally, JetA and JetD should be under the control of an inducible promoter. We decided on the GAL4.ER/Gal1 system²⁹, a fusion of the GAL4 transcription activator domain to the estrogen-receptor estrogen binding domain, which allows induction of elements under the control of the strong Gal1 promoter by β -estradiol addition. JetB and JetC should be under the control of the strong constitutive TEF promoter. Furthermore, NLS tags should be added to direct the Jet proteins to the nucleus, as well as a 3xV5 epitope tag to be able to determine expression levels. All should be stably integrated into the yeast genome.

Finally, we want strains that allow for straight forward determination off plasmid loss. Hence, we chose a strain background with the *ade2-AT49C* (*ade-fs1*) mutation, which carries a frameshift mutation (AT to C exchange) close to its translation start on position 49. The truncation of ADE2 causes the accumulation of a red pigment when the adenine biosynthetic pathway is active. Combining this with a target plasmid containing a functional copy of ADE2, allows us to screen for plasmid loss by the formation of red sectors in colonies.

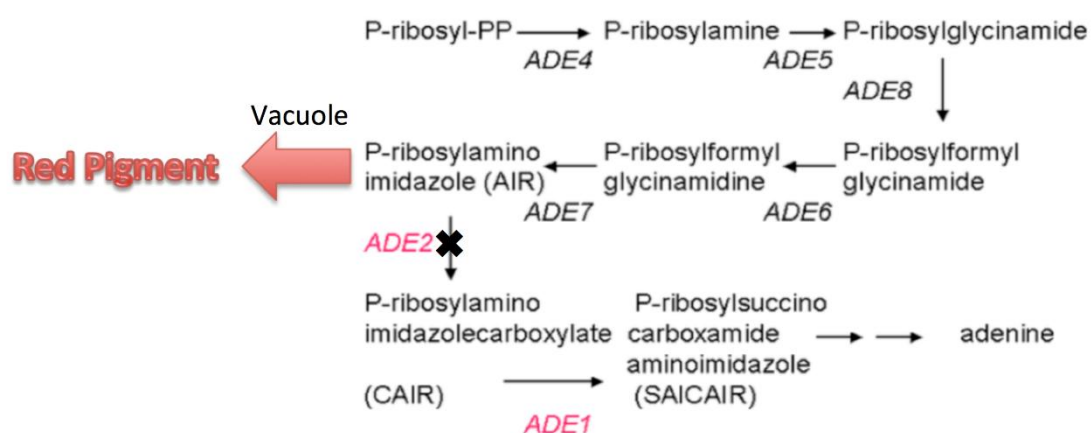


Figure 6 Adenine biosynthetic pathway in yeast and results of disrupting ADE2,

Copyright © 2001, Beth A. Montelone, Ph. D., GENE Project and Research Link 2000., modified

Type I WADJET system from *E. coli* was kindly provided by Stefan Gruber. We adapted the original constructs and arrived at two yeast integration plasmids p1113 for JetABC and p1109 for JetD (also versions without 3xV5 tag). We also varied the nucleotide sequence of two of the V5 tags on p1113 to make cloning more efficient.

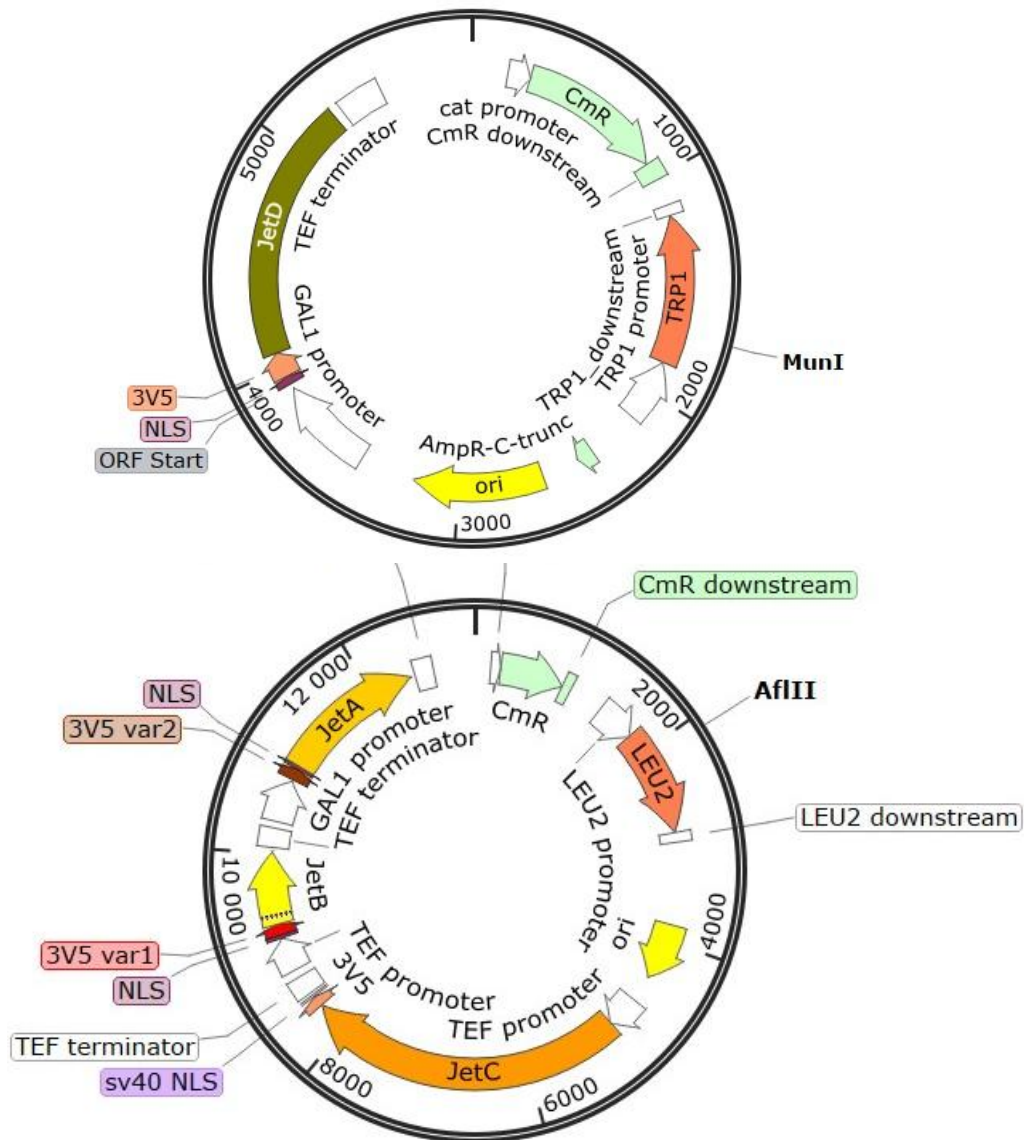


Figure 7 Top: Plasmid map of p1109 JetD, MunI site for linearization and integration into *trp1* locus marked. Bottom: Plasmid map of p1113 JetABC, AflIII site for linearization and integration into *leu2* locus marked

The Plasmids were linearized and integrated into strain 9381 (containing the *ade2-AT49C* mutation and *Gal4.ER*) to create strain 9490 and transformants were confirmed by checking both junctions via colony PCR.

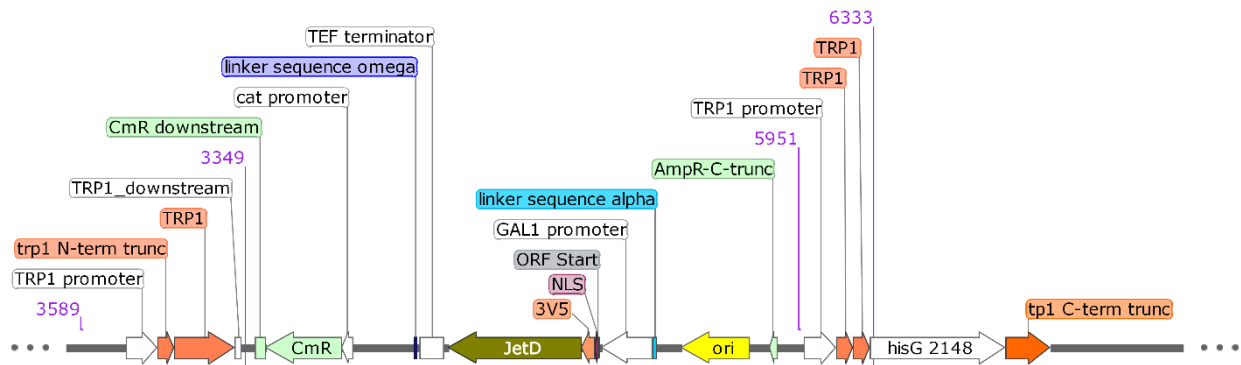


Figure 8 Map of the integration of p1109 into *hisG* disrupted *TRP1* locus following *MunI* linearization.

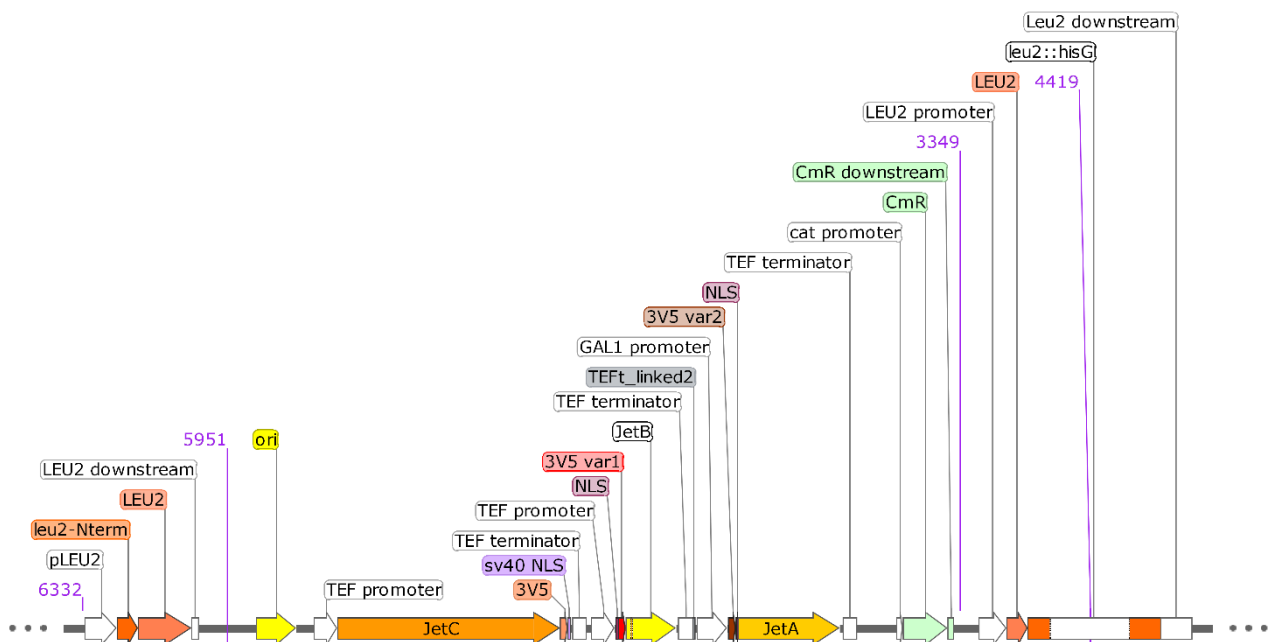


Figure 9 Map of the integration of p1113 into *hisG* disrupted *LEU2* locus following *AflIII* linearization.

3.2. Expression of single integration WADJET

Having constructed suitable strains, the next task was to analyse if and at what levels the WADJET proteins are expressed in yeast and if JetA and JetD can indeed be induced with β -estradiol.

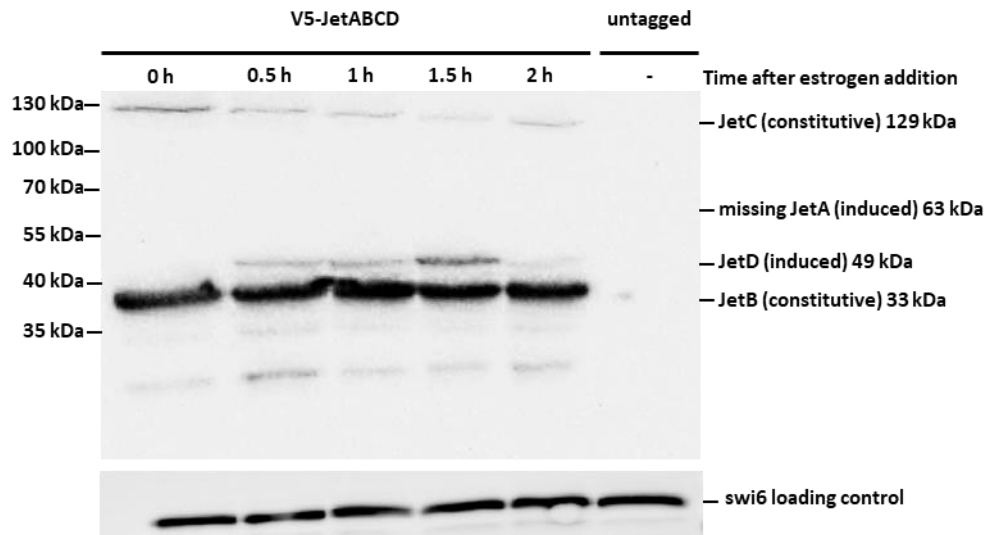


Figure 10 Western blot for expression of JetABCD with 3V5 tag in strain 9490. Blot was stained with primary antibodies mouse- α -V5(FK173, 1:2500) and rabbit- α -swi6 (FK133, 1:10000). Secondary antibodies were goat- α -mouse HRP (Fk75, 1:50000) and goat- α -rabbit starbright 700 (FK311, 1:3000). Culture was induced with $1\mu\text{M}$ β -estradiol at OD600=0.6. Strain 3086 was used as untagged control.

The western blot (**Figure 10**) shows four main bands. The topmost, weakly expressed band corresponds to the expected size of JetC (**Figure 10**). The singular inducible band fits with the JetDs expected size and is present already after 0.5 hours. There is also a very prominent band at around 40kDa which would be closest to the expected size of JetB, which with an expected size of 33kDa, is smaller than were the band segregates. Concerningly, a band corresponding to JetA is missing. Since JetA and JetD share the same promoter and terminator, we suspected that JetA is degraded at the protein level.

Hence the next task was to find conditions where JetA expression can be detected and conclusively assign the bands observed to the respective WADJET components.

3.3. Separate Expression of JetA and JetBC

JetA and JetBC were expressed from different constructs, both integrated into the LEU2 locus in separate strains. The strong band at around 40kDa seems indeed to be JetB. JetC expression is again very weak (**Figure 11**). JetA alone does not produce any detectable expression. The problem could potentially also lie in mRNA stability or in codon usage.

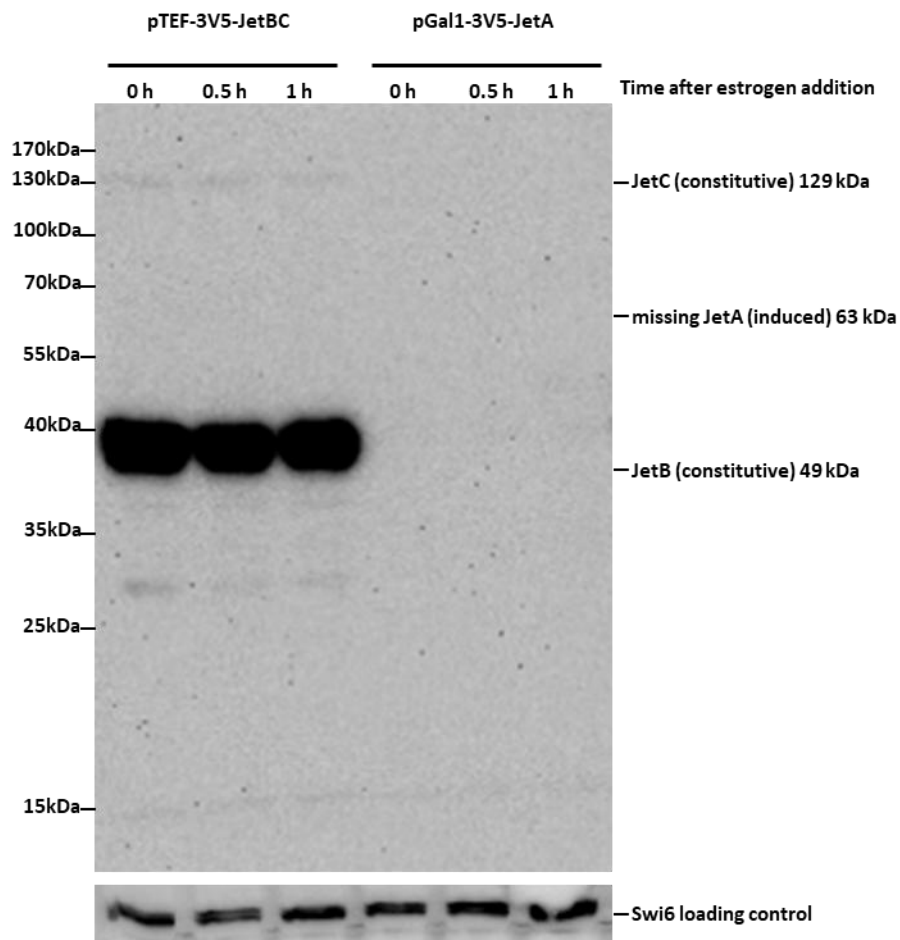


Figure 11 Western blot for expression of JetBC and JetA in separate strains (9527,9526). Blot was stained with primary antibodies mouse- α -V5(FK173, 1:2500) and rabbit- α -swi6 (FK133, 1:10000). Secondary antibodies were goat- α -mouse HRP (Fk75, 1:50000) and goat- α -rabbit starbright 700 (FK311, 1:3000). Culture was induced with 1 μ M β -estradiol at OD600=0.6.

To get an estimation of the influence of codon usage on the expression of WADJET components the Codon Adaptation Index or CAI for short can be consulted.³⁰ The values are calculated using a set of highly expressed proteins with their S288c and SK1 sequence respectively.³¹ Calculation is performed using the CAI and biophyton modules in python.

Table 1: Codon adaptation index (CAI) of WADJET proteins calculated with S288c and SK1 reference sequences

CAI	S288c	SK1
JetA	0.11	0.17
JetB	0.09	0.15
JetC	0.10	0.16
JetD	0.08	0.14

All WADJET components have a low CAI for both SK1 and S288C (**Table 1**). For reference, common bacterial proteins, not codon optimized, have a score from 0.1-0.2. Whereas highly expressed yeast genes like PGK1 or TDH2 score above 0.9 using this method and reference set. However, the Jet proteins have very similar scores amongst each other but wildly different expression levels. JetA has the best codon score within WADJET, but remains undetectable. This shows that suboptimal codon usage does not explain the failure of JetA expression. Nonetheless codon optimization may be helpful for the expression of WADJET components.

3.4. Overexpression of JetA and JetC

A straightforward way to increase protein levels is to express the components from a 2 μ multicopy plasmid. Expression of JetC is increased by roughly a factor of 5 when expressing it from a 2micron plasmid, however JetA remains undetectable (**Figure 12**, **Figure 13**). This further implicates degradation as the probable cause for its absence.

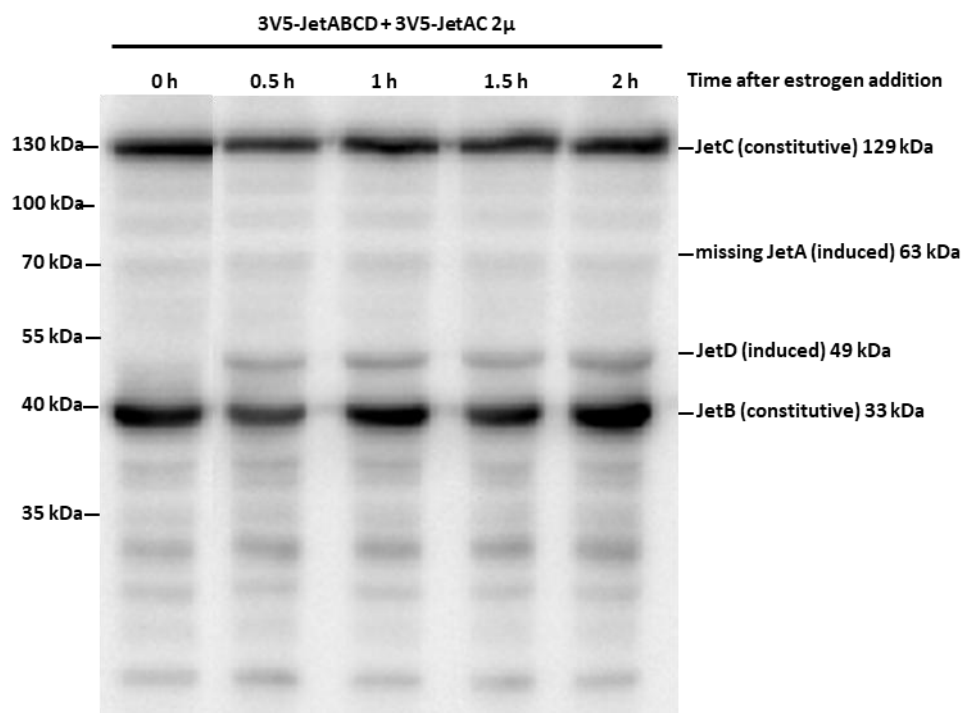


Figure 12 Western blot showing the expression of JetAC from 2 μ plasmid in JetABCD single integration background (Strain 9552). Blot was stained with primary antibody mouse- α -V5(FK173, 1:2500) Secondary antibodies was goat- α -mouse HRP (FK75, 1:50000). Culture was induced with 1 μ M β -estradiol at OD600=0.5.

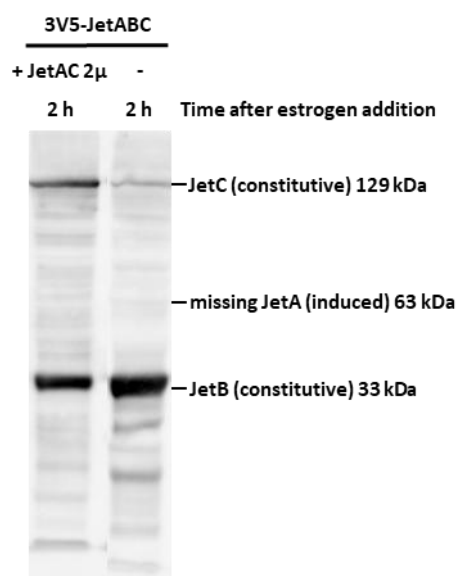


Figure 13 Western blot showing the difference in expression between JetC single integration (9560) without and with additional JetC expressed from 2 μ plasmid (9569). Blot was stained with primary antibody mouse- α -V5(FK173, 1:2000) Secondary antibodies was goat- α -mouse Starbright 520 (FK312, 1:3000). Culture was induced with 1 μ M β -estradiol at OD600=0.5.

3.5. Localization of WADJET Proteins

We wished to test whether WADJET Proteins were transported into the nucleus. Therefore, their localization was tested via *in situ* indirect immunostaining, making use of their common 3xV5 epitope tag. **Figure 14** shows that the signal overwhelmingly accumulates in the nucleus, confirming the functionality of NLS tags used. Judging from WB (**Figure 10**) the V5 signal is mostly comprised of JetB, with smaller contributions of JetC and JetD and JetA protein missing.

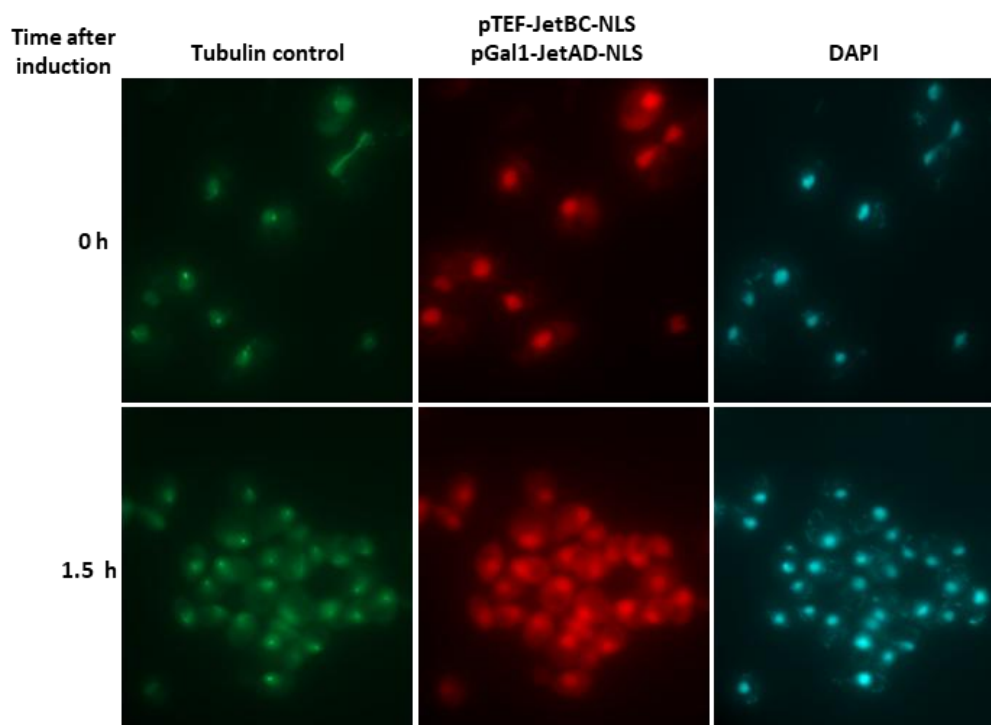


Figure 14 In situ immunostaining for localization of JetABCD with 3V5 tag in strain 9490. Stained with primary antibodies mouse- α -V5(FK173, 1:200) and rat- α -tubulin (FK26, 1:100). Secondary antibodies were goat- α -mouse CY3 (FK14, 1:200) and rabbit- α -rat FITC (FK18, 1:100). Culture was induced with 1 μ M β -estradiol at OD600=0.6.

3.6. Effect of proteasome inhibition on WADJET stability

Since we suspected that JetA is degraded, it could be stabilized by inhibiting the proteasome. For this purpose, we used the proteasome inhibitor MG-132. This experiment requires yeast strains with ABC transporter proteins deleted to prevent the cells from exporting the inhibitor.^{32,33} Coincidentally, MG-132 resulted in the appearance of a faint band roughly at the expected size of JetA. This faint band is however independent of estrogen, while JetA is under estrogen control. Additionally, some degradation products seem to be stabilized as there is more background in the lanes with MG-132 added (**Figure 15**). Inhibiting the proteasome also does not increase expression levels of any other Jet protein, but the abundance of ubiquitinated species is increased, indicating that MG-132 is working as intended. Thus, the proteasome seems not to be responsible for JetA's instability, so perhaps it could contain cleavage motifs that are recognized by a protease.

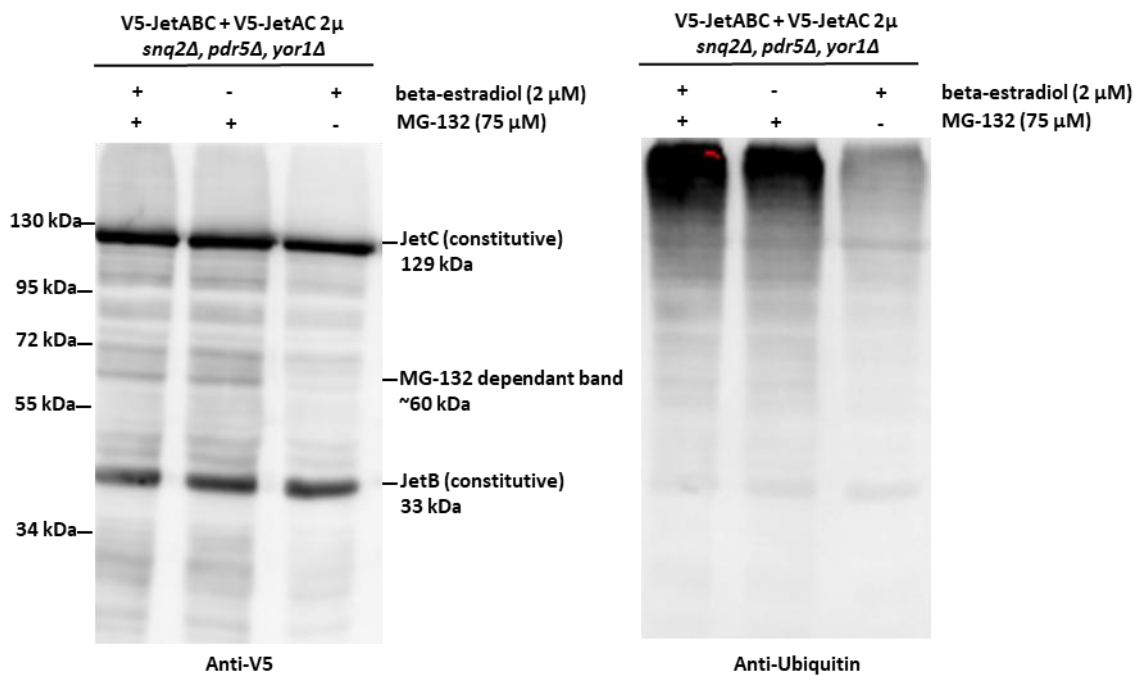


Figure 15 Left: Western blot showing the effect of MG-132 on the stability of Jet Proteins in strain 9569. Blot was stained with primary antibodies mouse- α -V5(FK173, 1:2500) and rabbit- α -k48 ubiquitin chain (CST catalog 8081 1:10000). Secondary antibodies were goat- α -mouse Starbright 520 (FK312, 1:3000) and goat- α -rabbit Starbright 700 (FK311, 1:3000). Culture was induced with 2 μ M β -estradiol at OD600=0.5.

3.7. KEX2 deletion is lethal in SK1

Since inhibiting the proteasome has no effect on the stability of JetA and the in-situ staining shows no apparent V5 signal accumulation in the vacuole, a protease might specifically recognize and degrade JetA. Resources providing predictions for proteolytic cleavage in yeast are limited. Using the online resource Eukaryotic Linear Motif (ELM) at elm.eu.org, we found 8 recognition sites, 4 of which were considered highly conserved, for the yeast protease KEX2 in the sequence of JetA (**Figure 16**). KEX2 is a serine protease involved in protein maturation of alpha factor, killer toxin and cell wall components.^{34,35} It is membrane bound, active at the ER and trans-golgi-network and cleaves after dibasic motifs (KR and RR).³⁴

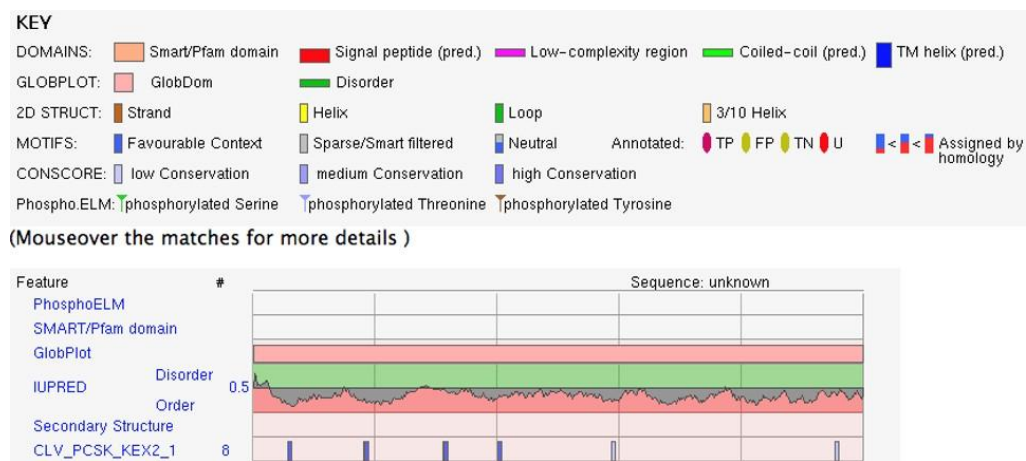


Figure 16 Prediction for cleavage associated yeast motifs contained in JetA sequence from <http://elm.eu.org/>

Deleting *kex2* might stabilize JetA enough to be able to detect expression. In the strain background S288C, the *kex2* null mutant is viable and exhibits increased sensitivity to certain chemicals as well as pH, but only slightly decreased growth rate.^{35,36}

However, we failed to obtain any haploid SK1 *kex2Δ* strain. Only when we transformed a diploid SK1 strain with a *kex2Δ* deletion cassette, we obtained colonies heterozygous for the deletion (**Figure 17**).

Upon dissection of the heterozygous *kex2Δ* strain, the two G418 resistant colonies expected for each tetrad were dead, while the G418 negative spores were alive, showing that the deletion is lethal in SK1 background. (**Figure 18**).

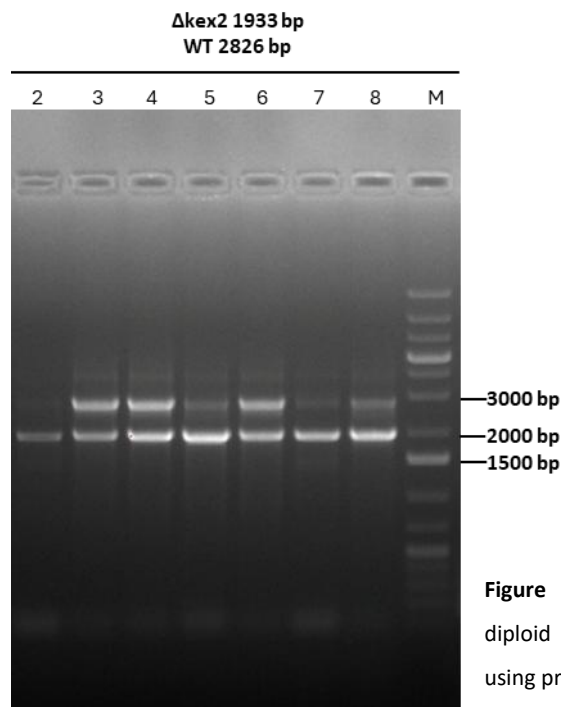


Figure 17 Colony PCR for the transformation of diploid strain 3088 with *kex2Δ::KanMX* fragment using primers 6577/6578

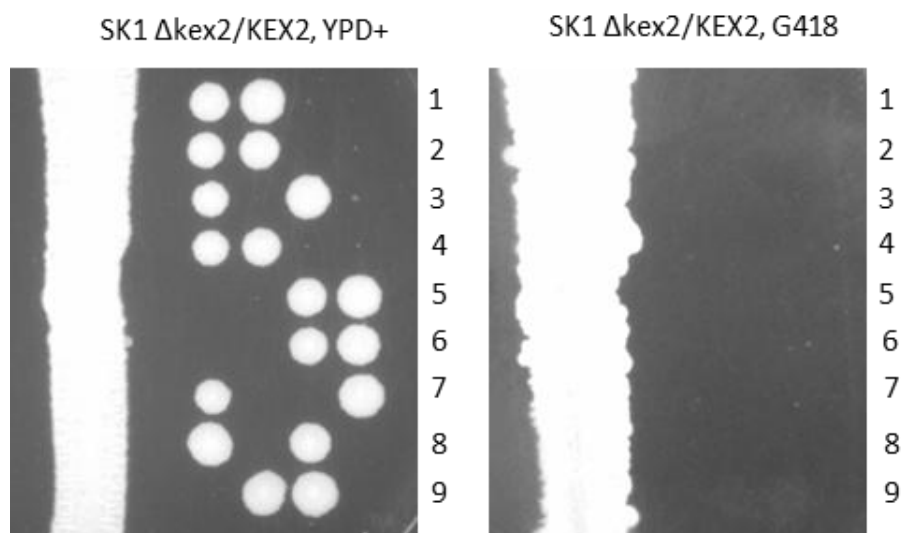


Figure 18 Left: Sporulation of 9585 on YPD+. Right: Colonies replica-plated onto YPD/G418.

Since *kex2Δ* is not lethal in S288c derived strains, a hybrid strain might be able to tolerate the deletion. A diploid BY4743 *kex2Δ* from the EUROSCARF deletion collection was tested for its viability in the haploid by letting it sporulate and dissecting five tetrads. Two spores of each tetrad are noticeably smaller (**Figure 19**), indicating that *kex2Δ* does affect growth in BY4743. After testing the viability of the spores, we crossed the sporulation mix directly with an SK1 strain containing *JetA* (9582). It was important to consider that the *KEX2* deletion makes strains of mating type *Mat α* sterile, therefore the *kex2Δ* strain must be *MATa* to ensure successful mating. After mating and dissection, we could isolate viable spores with the desired genotype. With five dead spores detected in six tetrads, there might be one synthetic lethal factor to *kex2Δ* in the SK1 (**Figure 19**).

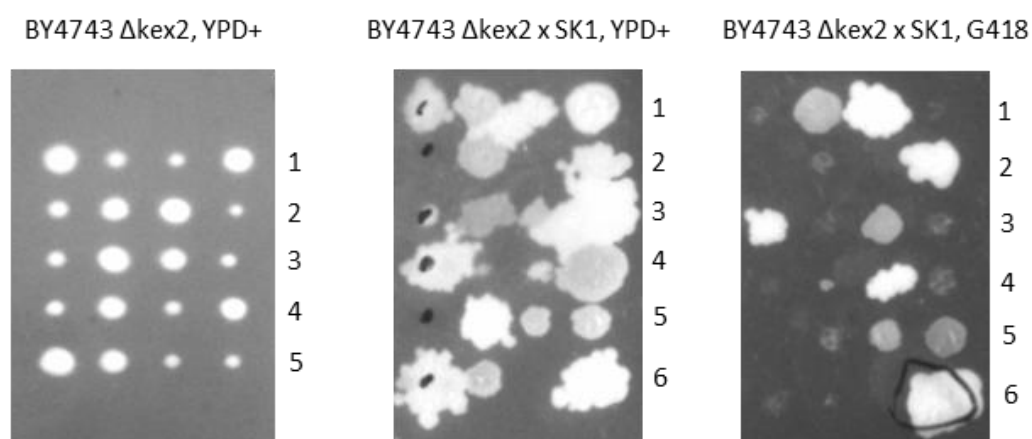


Figure 19 Left: Sporulation of FK9584 on YPD+. Middle: Sample Tetrads of 9584x9582 on YPD. Right: Tetrads from middle replica plated onto G418/Kanamycin.

3.8. Effect of *KEX2* deletion on JetA stability

Having isolated a suitable strain with the mixed SK1/BY background we could finally test the effect in western blot. It still shows no JetA signal in the *KEX2* deletion strain (**Figure 20**). Curiously, JetD became constitutive in the mixed background/*kex2* Δ strain, and its expression level was increased 2-fold compared to the control (**Figure 20**). Perhaps JetD is a substrate for Kex2. A second factor, which could also explain it becoming constitutive could be related to secretory stress in the deletion mutant. Since Kex2 is responsible for protein maturation, an accumulation of unprocessed proteins could lead to more chaperones, like hsp82, which is responsible for sequestering Gal4.ER, to be tied up in dealing with such unprocessed proteins.^{29,3738} This in turn could lead to estrogen independent, increased expression of JetD. Additionally, JetC is roughly 2-fold less abundant in the deletion strain, while JetB levels remain similar (**Figure 20**). This might be explained by a general inhibition of translation due to cellular stress caused by *kex2* Δ , which has disproportionate effect on the much larger JetC, since in yeast longer proteins are on average less abundant. Additionally, the mixed strain background might also play a role, since the two backgrounds show different preferences in codon usage.

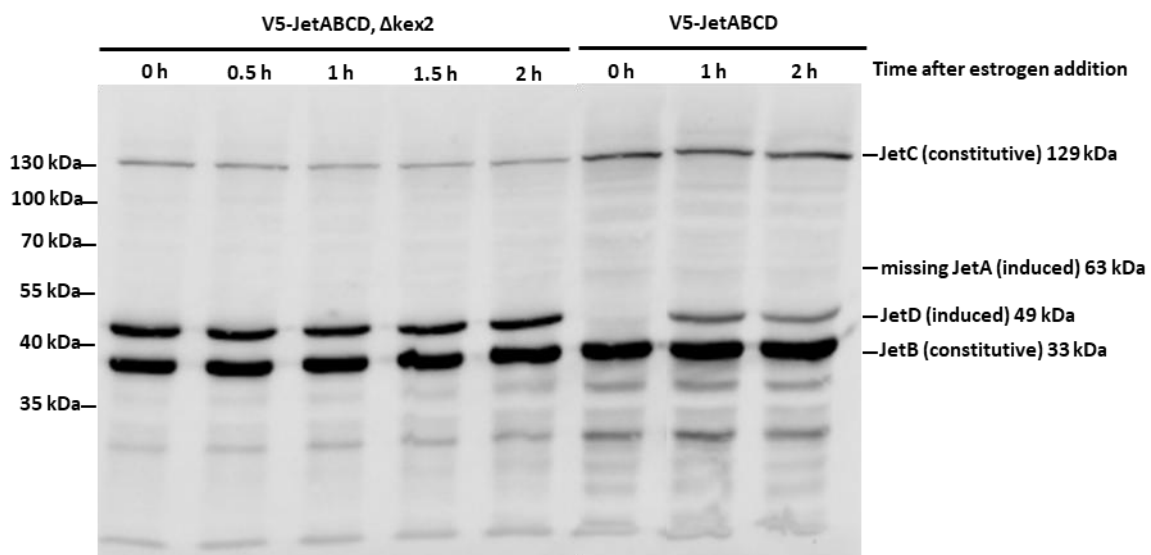


Figure 20 Western blot showing the effect of $\Delta kex2$ on the stability of Jet Proteins (Strain 9587, Strain 9490 control). Blot was stained with primary antibody mouse- α -V5(FK173, 1:2000) Secondary antibodies was goat- α -mouse Starbright 520 (Fk312, 1:3000). Culture was induced with 2 μ M β -estradiol at OD600=0.6.

3.9. C-Terminal Venus Tag stabilizes JetA

Since deleting *kex2Δ* had no effect on our ability to detect JetA, we tried to stabilize it by introducing a tag. We chose a C-terminal Venus tag which has a stabilizing effect on JetA, when expressed in *E. coli* (as observed by the lab of Stephan Gruber).³⁹ They show that adding a Venus tag to the C-terminus of JetA produces the largest yield among all tested constructs (**Figure 21**). Venus is a variant of the fluorescent protein YFP, containing several mutations that make it more tolerant towards acidic conditions, chloride ions and accelerate maturation at 37°C.⁴⁰

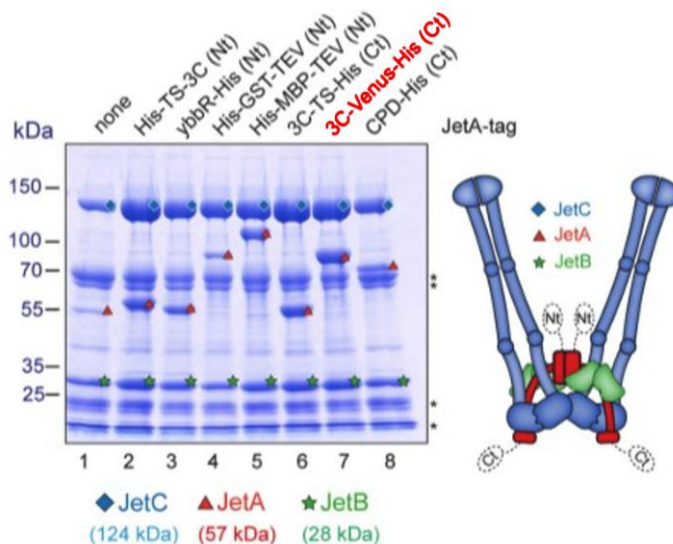


Figure 21 Expression screen of JetABC with various tags on JetA. Shown here is an SDS-gel of Nickel column purified protein. The bands in the untagged lane are due to JetC baseline affinity for Ni²⁺ resin. Adapted from Taschner et al. 2025.

We constructed strains, expressing JetA-Venus from a 2μ plasmid as well as from a single integrated copy. Indeed, this time a band of the expected size for JetA plus its tag appeared for both constructs (**Figure 22**). When expressing from a multicopy origin it has about 2x fold the expression level compared to JetD, however when expressed from a single copy, the expression is even below that of single integration JetC. Co-expressing JetBC from single integration did not increase the amount JetA-Venus and as a result we decided to proceed with expressing JetA-Venus from a multicopy vector. Having JetA on a plasmid requires us to select for it as well as it being a potential target for WADJET, should the system be active. Additionally, we do not know conclusively if JetA remains functional when fused to Venus. Despite these potential problems, expression of all four components in Yeast was achieved

for the first time. and allowed us to proceed to assaying if the system can indeed attack plasmids in a eukaryote under these conditions.

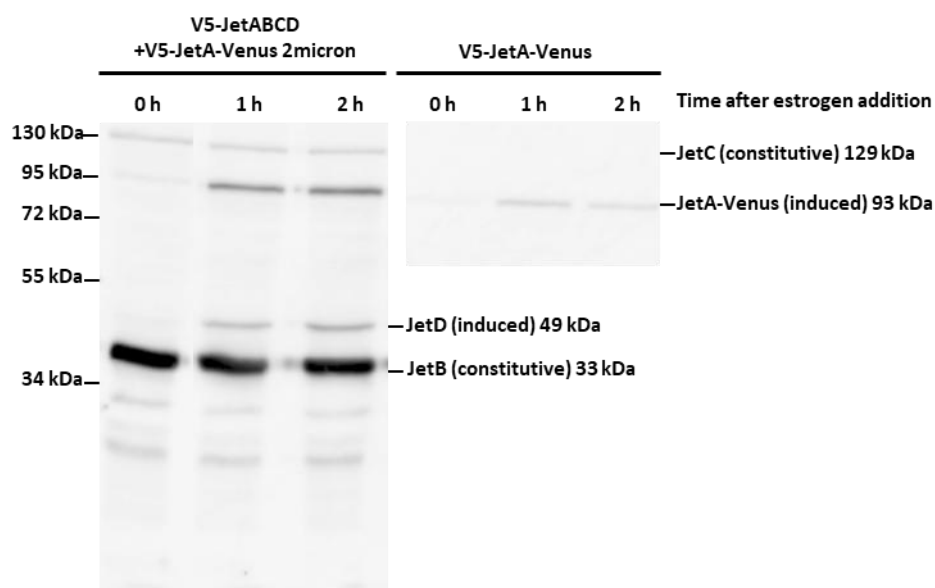


Figure 22 Western blot showing the effect of C-terminal Venus tag on the stability of JetA. Blot was stained with primary antibody mouse- α -V5(FK173, 1:2000) and secondary antibody was goat- α -mouse Starbright 520 (Fk312, 1:3000). Culture was induced with 2 μ M β -estradiol at OD600=0.7.

Exactly how the Venus-tag increases the expressions levels of JetA is unclear. The most likely mechanism would be preventing degradation or promote solubility. However, addition of MG132 showed no effect and since the C-Terminus of JetA consists of a winged-helix domain, a motif that is normally quite stable, it is unlikely that it works by masking degrons or disordered regions on the c-terminus.⁴¹ One possible explanation for the instability of JetA and why Venus might improve it, is intracellular pH. The intracellular pH of *E. coli* is slightly basic, depending on pH of the medium, while the yeast cytosol has a slightly acidic pH, again dependent on the medium.^{42,43} It is feasible that at this pH JetA aggregates and remains insoluble under the conditions of the protein extract preparation. Since Venus is stable under acidic conditions, it might increase solubility and probability of correct folding of JetA, thus preventing aggregation and making the protein detectable in Western blot. Arguing against this hypothesis is the fact that after induction of JetA, we do not see aggregates forming by *in situ* staining (**Figure 14**). Nonetheless, we tested for aggregation by adding either 8M Urea, 4% SDS (increased from to 2%) or both to the western blot GSD sample buffer.

We could not detect any effect on the signal of JetA under these denaturing conditions (**Figure 23**). These findings suggest that the increased expression levels Venus confers onto JetA may still be due to a degradation pathway, which is not affected by MG132.

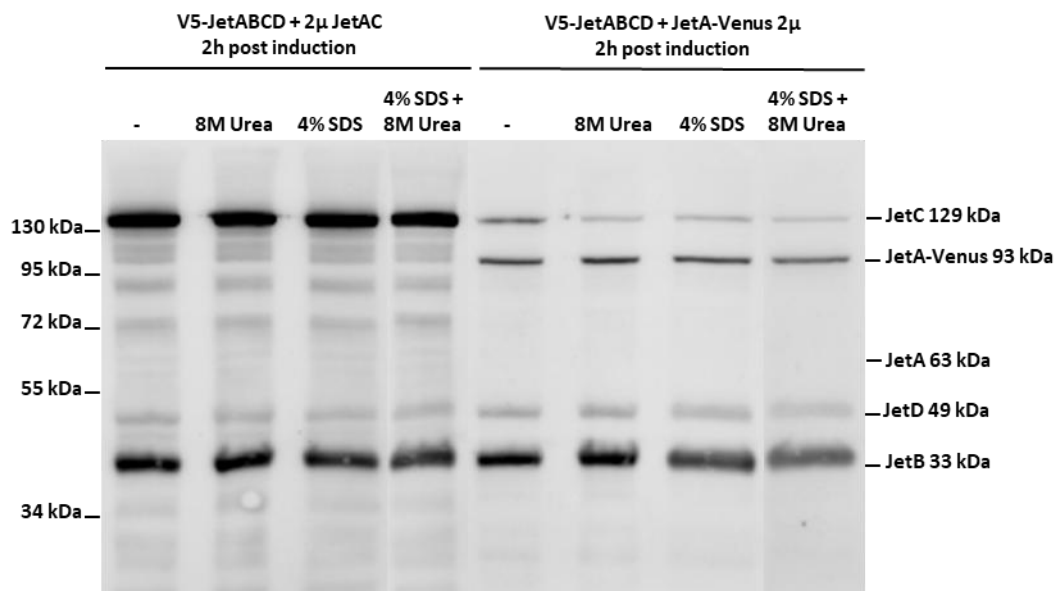


Figure 23 Western blot showing the effect additional denaturing condition on WADJET proteins (Strains 9552, 9582). Blot was stained with primary antibody mouse- α -V5(FK173, 1:2000) and secondary antibody was goat- α -mouse Starbright 520 (Fk312, 1:3000). Culture was induced with 1 μ M β -estradiol at OD600=0.5-0.7.

3.10. Plasmid stability assay

Having found conditions under which all WADJET components are expressed, we asked whether the stability of a resident plasmid is affected by inducing the system. We chose p1140 (**Figure 24**), containing a functional ADE2 gene, as the reporter to generate a colour change as readout. The color assay should sensitively result in red/white sector colonies.

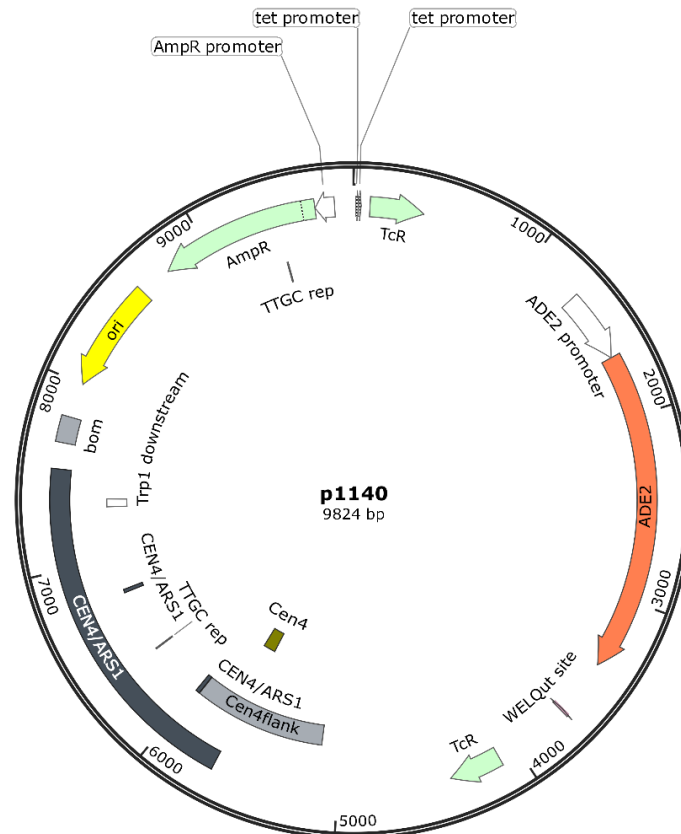


Figure 24: Plasmid map of p1140, single copy CEN plasmid as target for WADJET in color change assay

In preliminary experiments there was no difference between pre-induction of JetA/D in liquid medium and then plating on solid medium with estrogen compared to without pre induction. Plates were evaluated after 5 days by counting white, red and sector colonies. Red colonies represent cells without plasmid before WADJET induction. They do not represent WADJET-induced plasmid loss. In contrast, sector colonies, especially with multiple sectors represent cells that have lost the plasmid during growth under conditions where WADJET is induced.

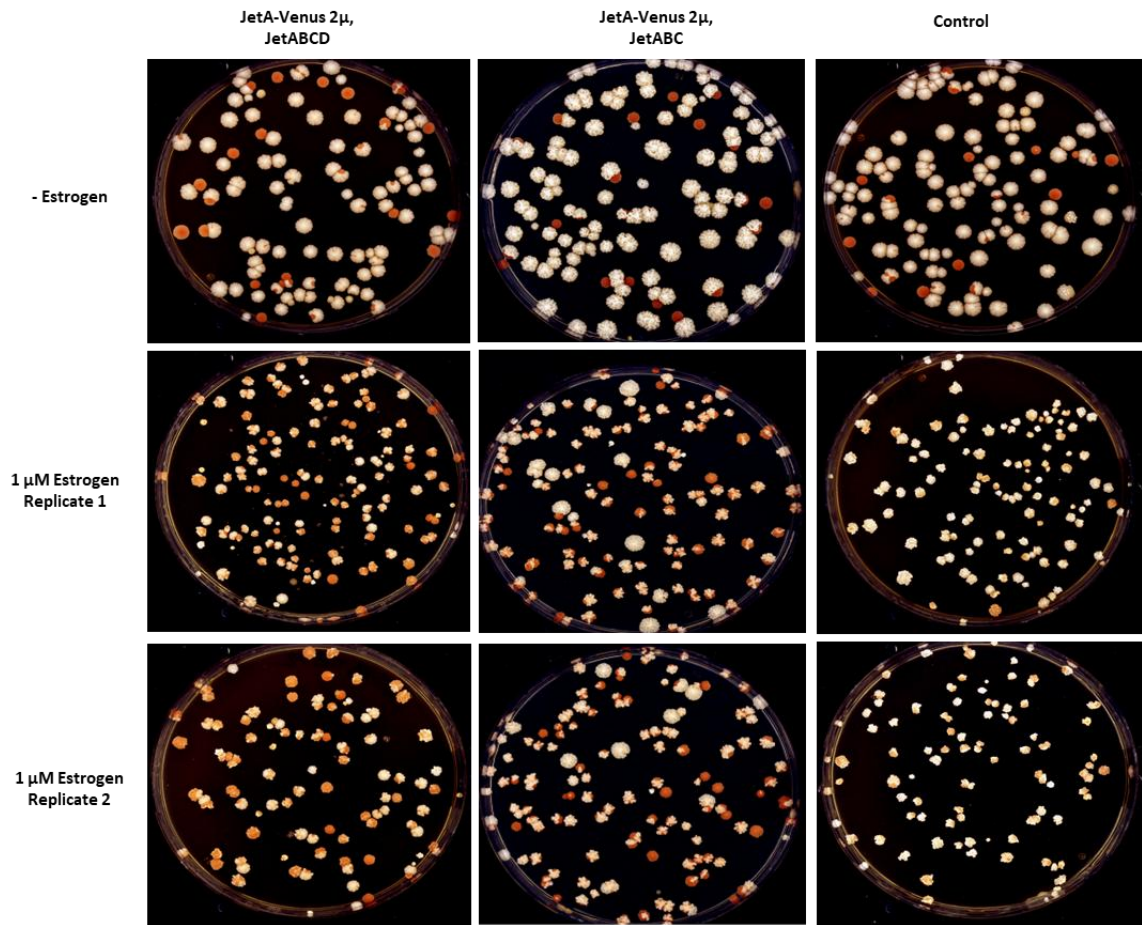


Figure 25: Left: Strain 9588 +/- estrogen Middle: Strain 9590 +/- estrogen Right: Strain 9595 +/- estrogen, shown one of two replicates

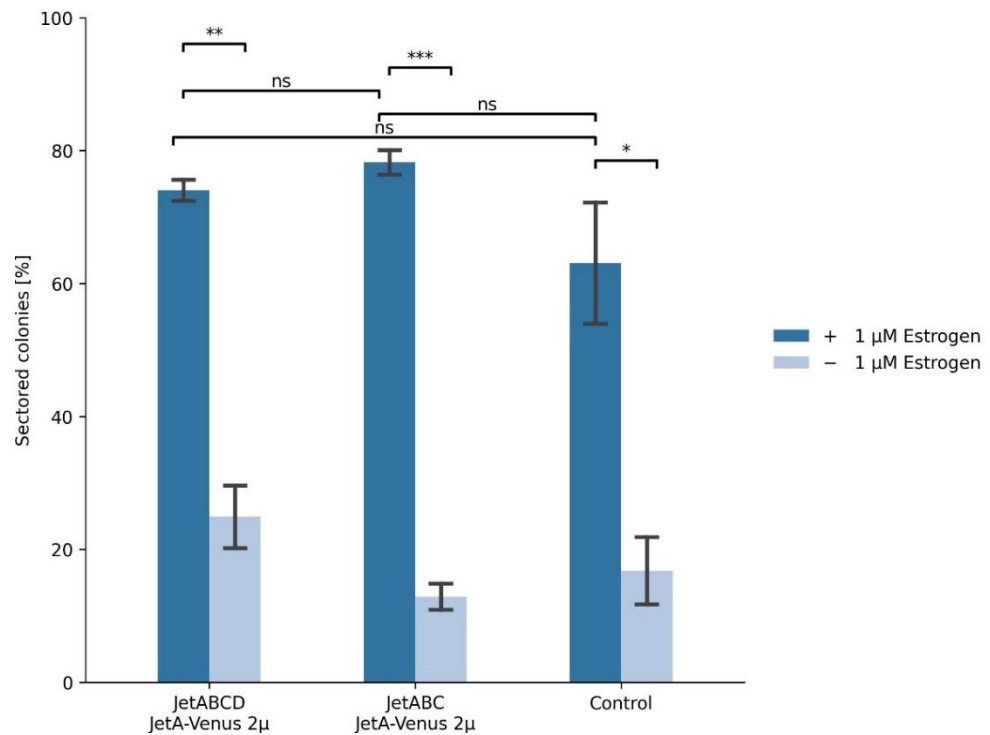


Figure 26: Quantification of two replicates +/- JetD (9588,9590) and control without WADJET (9595) of the plasmid stability assay. Statistical significance of differences between conditions was evaluated with independent t-test.

The presence of estrogen greatly increases the number of sectored colonies (**Figure 26**). However, it also does so in the control strain lacking the nuclease JetD, and even in the strain lacking all WADJET components. There are no significant differences in the fraction of sectored colonies between the strains (**Figure 26**). Curiously, the colour change is fainter in the presence of estrogen (**Figure 25**). However, in strains containing JetA-Venus, expressed from a plasmid carrying the HIS3 marker, the colour change on estrogen is more prominent than in the control but not as distinct as in the absence of estrogen, indicating that the effect of estrogen on the formation of the red pigment is somewhat alleviated by JetA or more likely by the presence of a functional HIS3. Colonies on plates with estrogen are also smaller indicating an effect on growth. This effect was only observed in strains containing the Gal4.ER.VP16 fusion protein, suggesting that is due to the activation of Gal4 regulated downstream effectors. In summary we found a strong, direct effect of estrogen on growth, pigment formation and plasmid stability in strains containing the Gal4/ER system, presumably due to overexpression of the Gal operon.

3.11. Transformation efficiency

A possible cause for why WADJET would fail to restrict plasmids in yeast, is the presence of nucleosomes on eukaryotic DNA. We tested the effect of WADJET on transformation efficiency, where naked DNA enters the cell and could be restricted before acquiring chromatin. When we added DNA to estrogen pre-treated cells (1 μ M, 2.5h) and selected on estrogen containing medium, we found that transformation efficiency was severely reduced independent of WADJET components. This mirrors the results we obtained in the plasmid stability assay and further confirms that the Gal4 based induction system is incompatible with both plasmid stability and transformation efficiency assays.

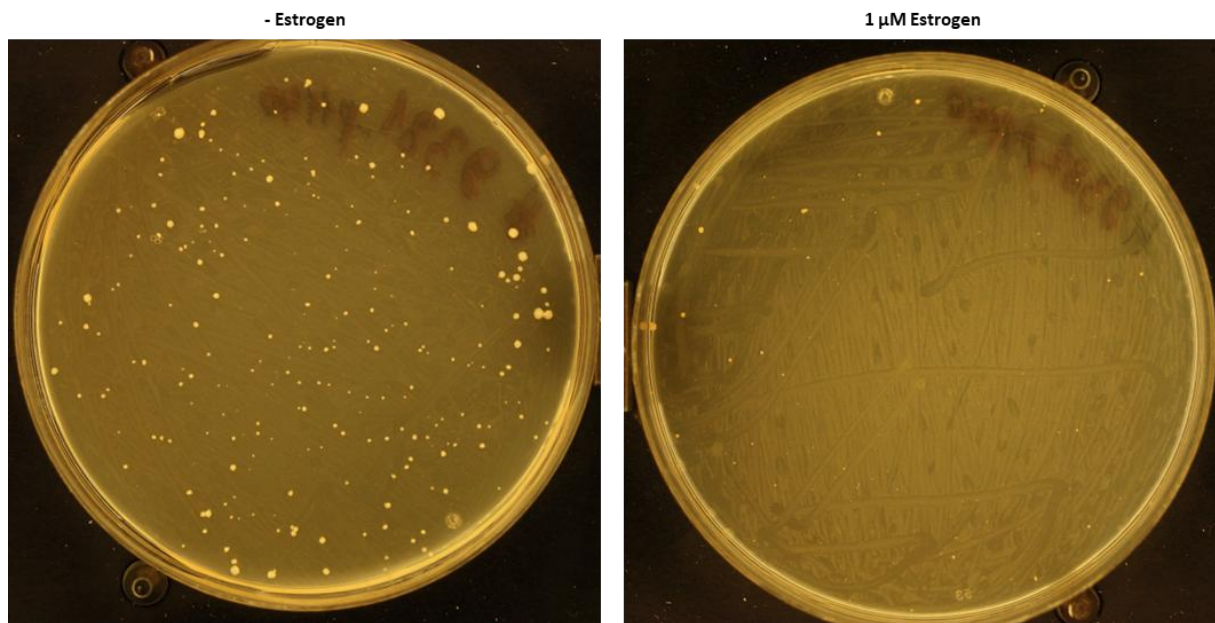


Figure 28: Left Transformation of control strain 9381 (no WADJET) with p1140 on -ADE without Estrogen. Right: Left Transformation of 9381 with p1140 on -ADE with 1 μ M Estrogen. $\sim 5 \times 10^7$ cells plated

3.12. Direct detection of cleavage via Southern blotting

Finally, potential cleavage by WADJET in yeast can be detected directly by Southern blotting, a physical method that does not require prolonged exposure to estrogen. For this purpose, the plasmid DNA, including potentially linearized species, must be isolated from yeast. One approach would be total genomic DNA extraction. However this technology prioritizes large genomic DNA, which would introduce unnecessary background, since we want to visualize plasmid DNA exclusively. Therefore, we tested the Yeast Miniprep II kit from Zymoresearch, which claims to specifically extract circular and linear plasmid DNA from yeast. The established Klein lab protocol for radioactive southern blot has a sensitivity in the order of 10^{-17} mol. However, we are aiming to use a non-radioactive method based on DIG-labeled probes and chemiluminescence. Given the same sensitivity, we would have to extract at least 5 ng of target plasmid DNA to be able to detect about 1% linearized species. The Plasmid yield was determined with qPCR and Qubit. We chose p1138, a 2 μ plasmid expressing JetA-Venus as the target plasmid. In addition, the WADJET system should also target the endogenous 2 μ plasmid that is present in wild type cells with an average of 50 copies. We designed primers for synthesis of southern blot DIG-probes and primers for qPCR to detect all 2 μ plasmids, as well as probes specific for p1138.

Table 2: Plasmid isolation yield for p1138 (strain 9581), endogenous 2 μ (strain 3086) and combined (strain 9581) as determined by qPCR (6579/6580 2 μ , 6583/6584 p1138) and Qubit.

	Concentration [ng/ μ L]
2 μ Qubit	10.4 $\pm$ 1.8
2 μ qPCR	6.3 $\pm$ 1.0
p1138 qPCR	16.4 $\pm$ 4.1
2 μ + p1138 Qubit	33.9 $\pm$ 6.7
2 μ + p1138 qPCR	30.6 $\pm$ 1.8

Qubit and qPCR are in reasonable agreement concerning total plasmid yield. These measurements showed that the plasmid isolation yield is likely sufficient for Southern blotting.

The experiments were performed in an *exo1Δ* strain background to avoid exonucleolytic digestion and to stabilize potential linear fragments.^{44,45}

First, we verified the expression of all WADJET proteins in the test strain, which shows inducible constant expression of JetA/D reached already after 30 minutes (**Figure 29**).

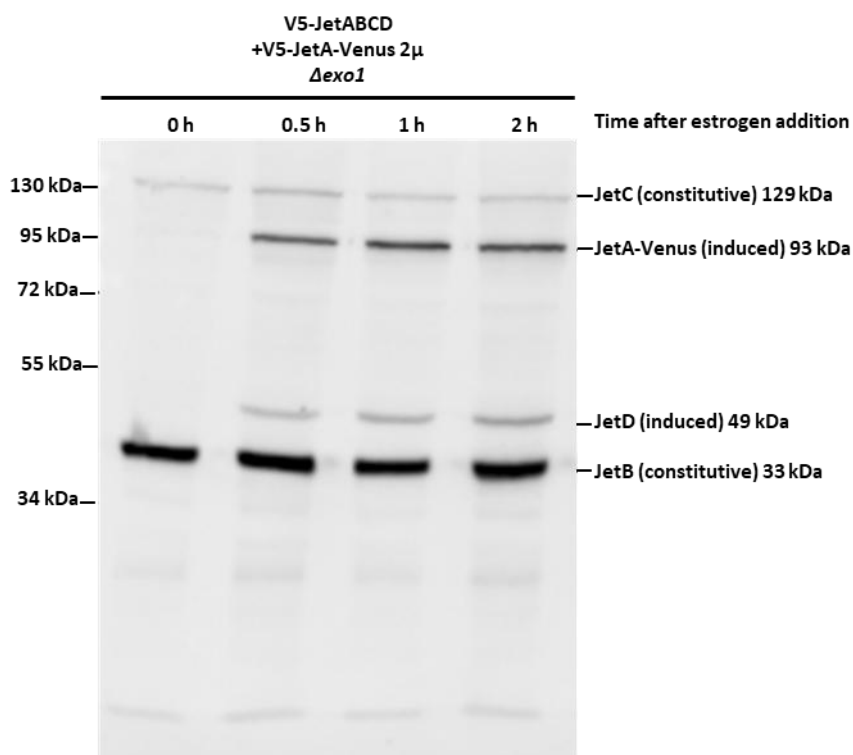


Figure 29: Western blot showing the expression of WADJET proteins in southern blot assay strain 9591 stained with primary antibody mouse- α -V5(FK173, 1:2000) and secondary antibody was goat- α -mouse Starbright 520 (Fk312, 1:3000). Culture was induced with 2 μ M β -estradiol at OD600=0.7.

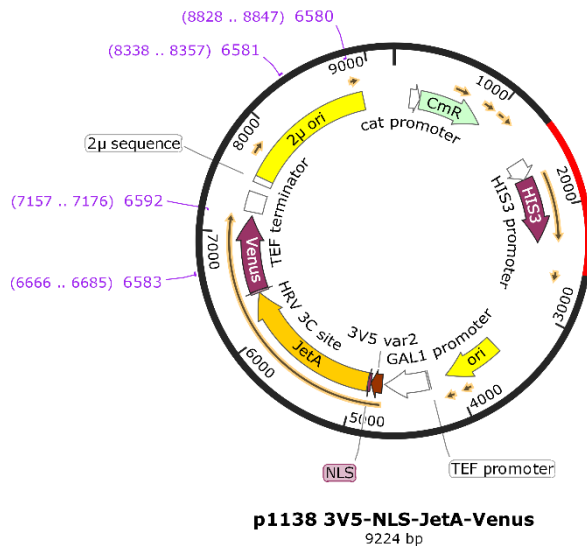


Figure 30: Plasmid map of p1138 illustrating binding of primer pairs to generate probes for southern blot. 6581 and 6580 bind in the 2μ origin. 6592 and 6583 bind in the region coding the venus tag and as such are specific for p1138.

We then used a probe targeting the 2μ origin to visualize all plasmids in the samples (**Figure 30**). Indeed, several different bands were detected in the samples (**Figure 31**), which we assigned, based on the control lanes containing pure 2μ or pure p1138, circular or linearized. The second from top band of the lane showing 2μ undigested may correspond to a supercoiled catenane created during replication, which disappears upon linearization. The nicked 2μ band runs at the same size as or close to the linear p1138.

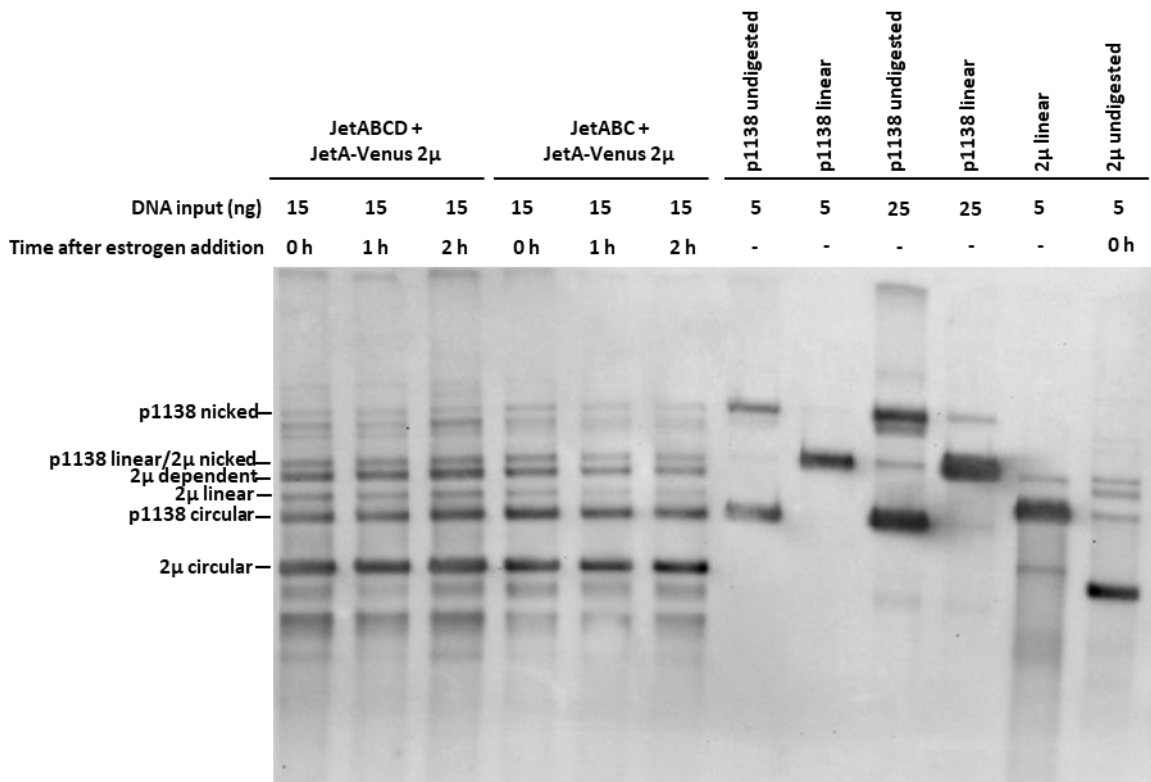


Figure 31: Southern blot using DIG-probe generated by primers 6580/6581 (500 bp) targeting 2μ origin (20ng/mL) in strain 9591.

To avoid miss-assignment between linear p1138 and a 2 μ species, we repeated the Southern blot using the p1138-specific probe (**Figure 30**). Unfortunately, 2 μ was still weakly detectable, but nevertheless interpretation was simplified. This time, only the supercoiled species of the 2 μ is visible in the assay, thus both linear and supercoiled/relaxed species of p1138 can be quantified. Quantification showed no accumulation of linear p1138 after induction (**Figure 32**, **Figure 33**). This analysis was less sensitive than expected, because of the unexpected presence of ~12% linear plasmid before induction. We do not know whether that may have been caused by DNA isolation procedure, but clearly, we do not see any increase of signal over this background after induction.

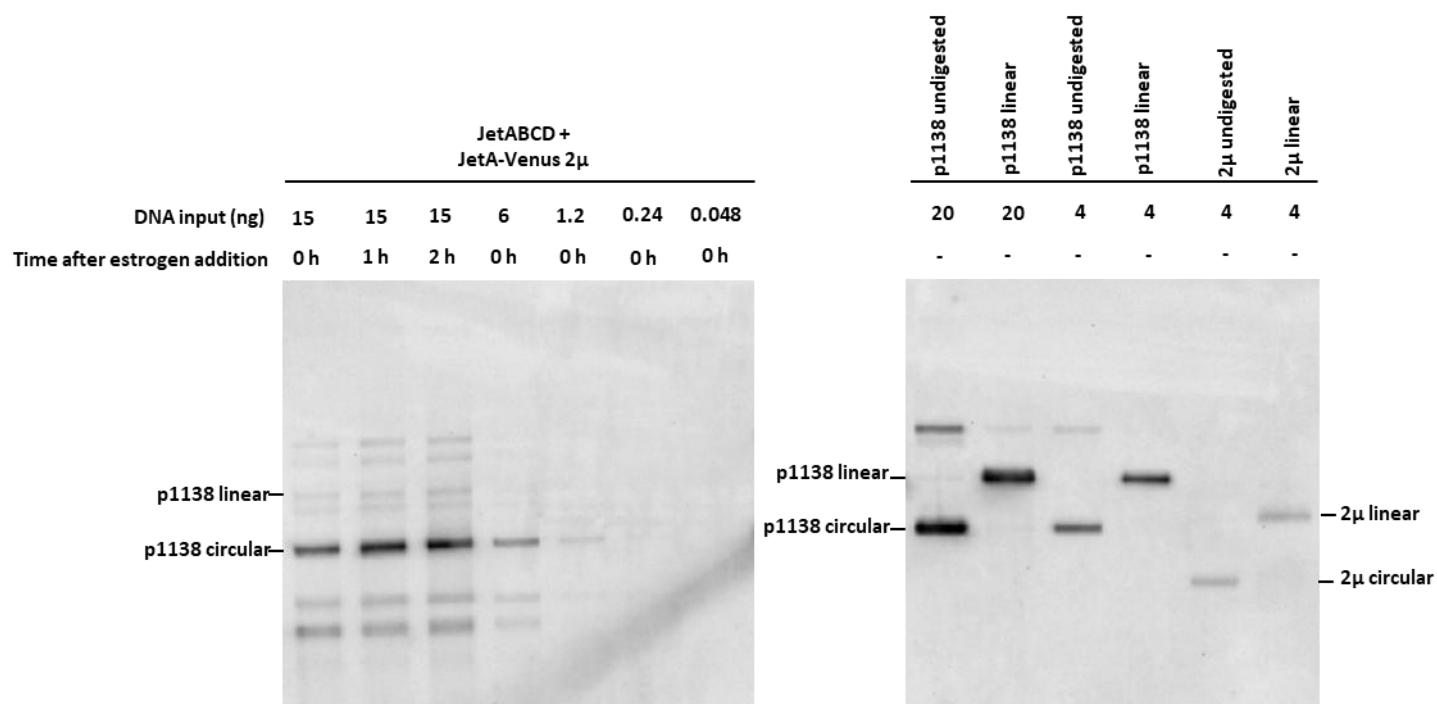


Figure 32 Southern blot using DIG-probe generated by primers 6583/6592 (500 bp) targeting p1138 (20ng/mL) in strain 9591.

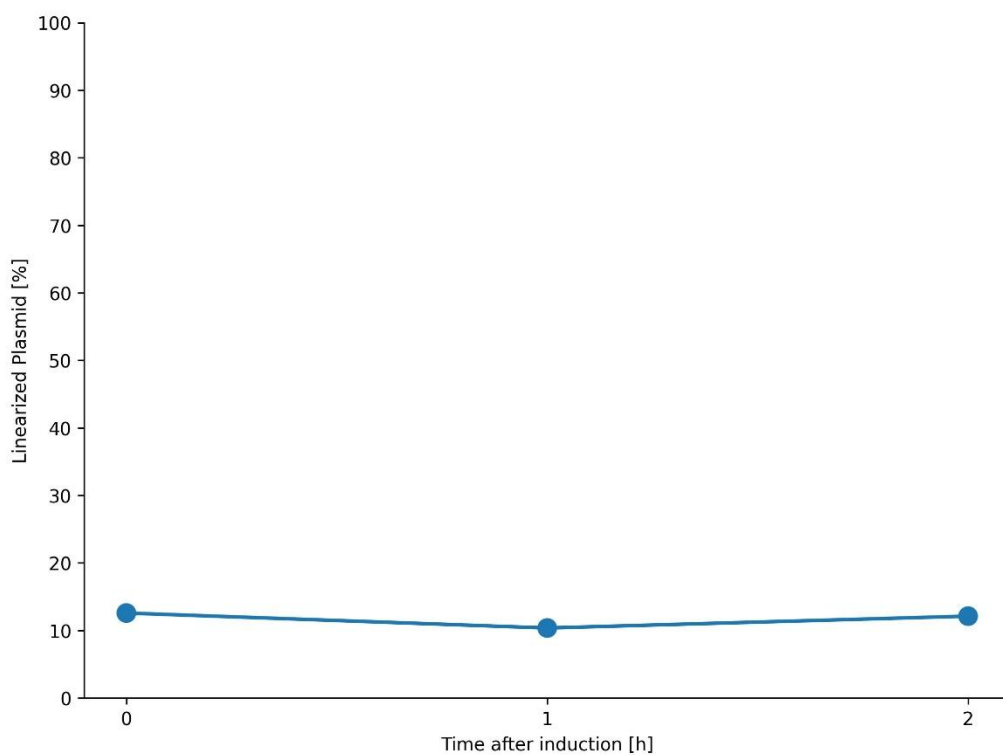


Figure 33 Quantification of p1138 cleavage southern blot, percent of linear species as fraction of total plasmid signal at each time point is shown.

4. Discussion

4.1. Expression of WADJET in yeast

Historically, studying bacterial defence systems has yielded tremendous results, with systems like restriction/modification enzymes and CRISPR/Cas being invaluable tools for molecular biology. Another new defence system from bacteria, WADJET, with its unique ability to detect and cleave DNA entirely based on circularity, shows the potential to be one such tool. For instance, it could be employed as an anti-viral agent. Since WADJET cleavage is sequence independent, this would make it much harder to adapt for viruses with a circular genome or those that employ circular DNA at any point in their lifecycles. Another use could be as an inducible plasmid clearance tool, when prolonged retention of a plasmid is not desired or for example to remove plasmids from certain strains or preparations. In any case WADJET would have to be studied in eukaryotic cells to take advantage of its potential. The simple, well understood model system *Saccharomyces cerevisiae* represented an ideal starting point for this endeavour. In the past, bacterial defence system could readily be adapted to eukaryotes. R/M enzymes, specifically EcoRI, have been shown to be functional *in vivo* in yeast, without modifying the protein itself.⁴⁶ In this case only the addition of a yeast promoter was required for expression of the bacterial enzyme. It was functional in the intracellular milieu of the yeast cell and was even able to enter the nucleus without NLS.⁴⁶ The story was much the same for the CRISPR/Cas system. Cas proteins could be stably expressed in yeast, under control of a suitable yeast promoter.^{47–49} In this case, NLS sequences were required for Cas proteins to enter the nucleus and codon-optimization for improved expression.^{47–49} Again the yeast cellular environment did not abolish enzymatic activity. This is not unexpected since yeast and bacteria like *E.coli*, share similar ion conditions and pH.^{50–52} These two examples serve as precedent that in the past, studying bacterial immune systems in eukaryotes has been immensely valuable and that expression of functional proteins in yeast was possible without many obstacles. We found the situation to be less straightforward with WADJET. Our findings concerning expression of the protein complex will be discussed here, whereas functionality will be treated later in **4.4**.

We could not detect any JetA expression from the Gal1/10 promoter, and JetC expression is weak. We found that expression of JetC from a 2 μ plasmid, increases protein levels 5-fold

whereas for JetA, expression from a multicopy vector was insufficient to get detectable signal. Inhibiting the proteasome with MG132 had no effect on the abundance of full-length Jet proteins or their potential proteolytic products. Kex2 is a yeast protease with numerous cleavage motifs contained in JetA, yet deletion of Kex2 did not recover JetA signal. This suggests that Kex2 is either not or redundantly acting on JetA, despite the presence of these cleavage motifs. We could finally achieve expression of JetA by fusing it with a Venus tag⁴⁰ at the C-terminus. How exactly Venus confers stability to JetA is unclear, one possible explanation was by potentially preventing aggregation. However, *in situ* fluorescence showed no indication of aggregates after inducing JetA (**Figure 14**). Nonetheless we tried adding urea to the protein extracts to improve solubility of potential JetA aggregates, but this also did not yield an increase in signal. This suggests that a different degradation mechanism is at play. It is known that in yeast MG132 does not affect growth rate very much^{32,53}, as we have observed as well, likely because it mainly inhibits the chymotrypsin-like subunits of the proteasome, leaving other subunits active.³² It is possible that tryptic or caspase-like recognition sites on JetA are the cause for its instability. Future attempts at characterizing WADJET in yeast might try to inactivate Pup1 and Pre3 subunits, required for trypsin-like as well as caspase-like cleavage respectively⁵⁴. If this does also not yield increased expression levels for JetA, mRNA levels should be checked. Certainly, using codon optimized versions of the WADJET proteins would be advantageous. One could also test WADJET systems from organisms other than *E.coli*, which might be more suitable for expression in yeast.

4.2. kex2Δ is lethal in SK1

To test the role of *KEX2* in JetA instability, we deleted the gene. However, *kex2Δ* proved lethal in the SK1 strain background, which was not recorded in [SGD](#). Thus, we constructed an SK1/BY hybrid strain that can tolerate the deletion. While JetA expression remained undetectable in this strain, other changes in the expression of the WADJET components were observed. For one, JetD expression, under the control of pGAL1, was markedly increased and became constitutive. This is very difficult to explain, since the estrogen – GAL4 system worked perfectly inducible, as advertised in the *KEX2* proficient sister strain. One explanation could be that the absence of *KEX2*, which is involved in maturation of various proteins, may cause chaperones, like hsp90, which in turn is involved in sequestering Gal4, to be occupied with misfolded proteins, thereby allowing it to activate transcription independent of estrogen.^{29,37,38} It is also

possible, that it is a consequence of certain allelic combinations between SK1 and BY backgrounds.

4.3. Estrogen effects plasmid stability, growth and pigment formation in susceptible strains independent of WADJET

We aimed to determine the effects of WADJET on transformation efficiency and plasmid stability *in vivo*. Our experiments show that the system we chose for induction of JetA and JetD, has an independent effect on growth, pigment formation in *ade2Δ* mutants and plasmid stability. We observed a large fraction of colonies with decreased size after prolonged exposure to estrogen for strains containing GAL4-ER. Additionally, the colour change was fainter in the presence of estrogen as compared to without. The number of colonies with red sectors was greatly increased by the presence of estrogen in strains with and without WADJET. Lastly, transformation efficiency was also almost completely abolished by estrogen in GAL4-ER strains. These effects are most likely caused by broad activation of the GAL-regulon and pleiotropic effects of GAL4-ER. It is known that the constitutive expression of the genes controlled by Gal4 has a negative effect on growth⁵⁵ in the absence of galactose. We suspect that this could perhaps slow down accumulation of red pigment, but it was completely unexpected for us to see the activation of the Gal operon to cause plasmid instability and to curb transformation ability. It would be interesting to characterize this further and establish the unexpected connection between galactose metabolism and plasmid maintenance. Estrogen itself is nontoxic for yeast and causes no growth defect when Gal4-ER is absent.

4.4. Expression of the complete WADJET system in the yeast nucleus did not produce detectable plasmid cleavage

In addition to indirectly assaying the effect of WADJET in yeast via plasmid stability we also assayed plasmid cleavage directly by Southern Blotting. When testing cleavage of the 2 μ plasmid or a 2 μ derived selectable plasmid *in vivo*, we did not find an accumulation of linear plasmid up to 2 hours after induction of the WADJET system. Unexpectedly, we found a strong background signal of ~12% linearized plasmid independent of the WADJET system. This could be due to the activity of Flip recombinase encoded by the endogenous 2 μ , which makes single strand breaks at FRT sites contained in 2 μ plasmids.⁴ It could also be an artifact caused by the

plasmid isolation procedure or a combination of both. Nonetheless, there was no detectable change in the relative intensities of linear plasmid. We believe that 3-5% of cleavage should have been easily detectable based on the stability of the observed signal at around 12%.

Future experiments should start out testing the functionality of all tagged WADJET components *in vitro* and *in vivo*. In *E.coli* numerous tags have been tested and have all been shown functional. Nevertheless, this point should be clarified for the tags used here before concluding WADJET activity is inhibited in yeast.

If indeed WADJET is inactive in yeast, several possible explanations come to mind. First, the SMC complex may fail to assemble in yeast. Future experiments may test binary interactions by Co-immunoprecipitation. Complex formation could further be assayed by large scale expression and size exclusion chromatography or labelling each component with a different fluorophore or epitope for immunofluorescence and then observing if they co-localize. Another factor might be intracellular ion and pH conditions. They are however very similar in yeast and *E.coli* as well as in buffer conditions for biochemical WADJET assays.^{14,50-52} If the proteins assemble correctly the most likely reason for WADJET's failure to cleave would be eukaryotic chromatin. One way to address this might be to repeat the plasmid transformation assay in an expression system different from estrogen/Gal4, where transformation rates are unaffected by the induction system, for example CUP1 and plus/minus nuclear localization sequences, to potentially eliminate DNA in the cytoplasm. One might also use *E.Coli* with its native WADJET system and test, whether expression of nucleosomes in bacteria, which are known to assemble on bacterial DNA⁵⁶, inhibit the system in bacteria. Evidence arguing against chromatin abolishing WADJET cleavage was found by the Gruber lab. They show that, *in vitro*, WADJET can bypass roadblocks up to micrometre size and even when blocked, cleavage still occurs at the position of the roadblock.²⁸ Additionally, eukaryotic SMC complexes have also been shown to be able to bypass large obstacles.⁵⁷ But even if WADJET loop extrusion is not abolished by the presence of nucleosomes, they might still introduce other problems. The cleavage competent state of DNA extruded²⁸ by WADJET might not be possible to achieve on chromatinized DNA. It is known that chromatin has different mechanical properties which might well prevent JetD recruitment and cleavage.⁵⁸ Experiments, where plasmid DNA is precipitated via the V5-tag of WADJET, sonicated and ligated to sequencing adapters could determine whether proteins bind but are unable to loop extrude/fail to recruit JetD. If the cause for WADJET's inability to restrict plasmids in yeast is the failure to loop

extrude, one might try to create fusions between yeast SMC proteins, which are capable of extrusion and the bacterial complex, which can cleave upon reaching the end of the circle. In summary, this work has succeeded in the unexpectedly difficult task to express all components of the WADJET complex in a eukaryote, *S.cerevisiae*. While *in vivo* activity could not be observed, we learned many valuable lessons that may prove helpful in further pursuing the establishment of a eukaryotic DNA ring destroyer.

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6. Appendix

6.1. Primers

FK collection number	Sequence 5' to 3'
735	aattggtgtttggccgagc
2995	TTTGCAATCAATCTTTCTGTGGCGCAAACACCGACAGCTGgcacatttccccgaaaagtg
3349	TCCTTACGCATCTGTGCGGTAT
3589	GGAGCAGATGACGAGTTGGT
4079	aagttcttgaaaacaagaatc
4235	GGTAGTTATATAGGATGATCCTCTGGCATGAGATGTG
4419	GGTTCTGTCTGAAGTCGCGCC
5134	gctcggccaaacaaccaatt
5763	gcctttttacggttcctg
5948	CGTCAAGACTGTCAAGGAGGGTATTCTGGGCCTCCATGTCggcatccgcttacagac aag
5951	gcccagatgcgaagttaagtgcgcagaaaagtaatatcatgtggcttaactatgcggcatc
6151	GGCTAGTCCGTTATCAACTTGAAAAAgtggcaccgagtcggGAAAAGGAGCCATT AACG
6283	ctgtcacccgctcggcggcttctaattccgATCAATGCCGTAATCATTGACC
6323	CCGAGAAGGAGAAGTATTAC
6324	GTTGATAGCGAATGTAGACC
6325	TATGTTGGGTGGAAGTGAAGG
6326	CGTTTGTCTAGCAGAGGAATC
6332	AACTCCGGTATTACTCGAG
6333	tcatgcaccactggaagatc
6396	GTTGCTACTGGTGGACACCCGACTATCTACAGTAAGGAACgatcgatgaattcgagctcg
6397	CACTACGTCATCCTCATCATTTGAATGCGAACCCTCATctgagcagcgtaattctggaa c
6398	CAGTACTGGCGTGTGCTATAG
6399	TTTAAGGGAGACGACGATGC
6404	TCTATACTTTAACGTCAAGGAGAAAAAACCCCGATTctagATGGCGCCAAGTA AGAAGC

6405	gATGGCGCCAAGTAAGAAGCGGAAGATTTTTAAAggatccGGTAAACCTATACCT AATCC
6406	AAGCTTTTCACGCAATTCATCTGGGGAATACATGGCTGTACTATCCAGTCCTAA TAGAGG
6407	TACACCAATTCCTAATCCTCTATTAGGACTGGATAGTACAGCCATGTATTCCCCA GATGA
6408	atacactccgctatcgctac
6409	gATGGCGCCAAGTAAGAAGCGGAAGATTTTTAAAggatccGCCATGTATTCCCCA GATGA
6410	AACGCCTTGATGAAGACTGG
6411	cTTcTTAGGCGCCATtccCGCGTTCCCCGTCGCTGGTAGTGTCTGTTCAAGCTCTT CCCA
6412	ctagtgcctattcttagctagtgcctaggaAACTTTTCGTTTcTTcTTAGGCGCCATtcc
6413	AAgAAgAAACGAAAAGTTtcctaggcactagctaagaataggcactagcaggtTCAGTAC
6414	tTTCTTtgaaGGCGCCATcGGTTGTTTATGTTTCGGATGTGATGTGAGAACTGTATC CTAG
6415	AGGATACAGTTCTCACATCACATCCGAACATAAACAACCgATGGCGCctcaAAG AAaCG
6416	gATGGCGCctcaAAGAAaCGtAAaATcTTcAAgggttctGCGGGCTTTTTTGACAAG CT
6417	TCCGCTTCTTACTTGGCATGctagAATCCGGGGTTTTTTCTCCTTGACGTTAAAGT ATAG
6418	CTATACTTTAACGTCAAGGAGAAAAAACCCGGATTctagCATGCCAAGTAAGA AGCGG
6419	agCATGCCAAGTAAGAAGCGGAAGATTTTTAAAggatccGCCATGGAAGAAAAC ACAAGG
6420	CCAGTTCAGCTAAACCAACG
6421	TGACGGTGCAGGGATTAATG
6422	ATGTTTCGAGATCGTGAGCC
6423	GGATGCTTGTGAACGAAACC
6424	GGATTAGGTATAGGTTTACctccCGCGTTCCCCGTCGCTGGTAGTGTCTGTTCAA G

6425	AGAGCTTGAACGACACTACCAGCGACGGGGGAACGCGggaGGTAAACCTATACCTAATCC
6426	CTTTTCGTTTcTTcTTAGGCGCTGTACTATCCAGTCCTAATAGAGG
6427	CACCAATTCCTAATCCTCTATTAGGACTGGATAGTACAGCGCCTAAgAAgAAACGAAAAG
6428	GCCATCCAGTCCTAACAGGGGGTTTGGTATAGGCTTACCagaaccTTgAAgATtTTaCG
6429	GGTAAGCCTATACCAAACCCCCTGTTAGGACTGGATGGCAAACCGATTCTAACCCTGTTGGGTCTTGACTCAACTCC
6430	GGTACTGTCGAGACCTAAAAGGGGGTTTGGTATGGGAGTTGAGTCAAGACCCAACAGTGG
6431	AACTCCCATACCAAACCCCCTTTTAGGTCTCGACAGTACCGCGGGCTTTTTTGACAAGCT
6432	TTCCCATCAAGGCCCAATAGTGGATTCTGGGATGGGTTTTCCCATGctagAATCCGGGGT
6433	GGAAAACCCATCCCGAATCCACTATTGGGCCTTGATGGGAAGCCTATTCCAAATCCCCTGTTAGGTCTCGACTCTACTCC
6434	caTGgTGCTATCCAGTCCTAAAAGTGGGTAGGGATCGGAGTAGAGTCGAGACCTAACAG
6435	CCATGGCggatccTTTAAAAATCTTCCGCTTCTTACTTGGcaTGgTGCTATCCAGTCTA
6436	GCTGCCTTGTGTTTTCTTCC
6437	ACCAGCGACGGGGGAACGCGggaATGGCGCCTAAgAAgAAACGAAAAGTTtcctaggcac
6438	CCGCagaaccTTgAAgATtTTaCGtTTCTTtgaaGGCGCCATcG
6439	AGGAGAAAAAACCCCGGATTctagCATGCCAAGTAAGAAGCGG
6440	TTTTGCCAGAAGCTGCAACG
6448	AACTCCCATACCAAACCCCCTTTTAGGTCTCGACAGTACCGCGGGCTTTTTTGACAAGCTGATTAACCGGAGC
6449	TTCCCATCAAGGCCCAATAGTGGATTCTGGGATGGGTTTTCCCATGctagAATCCGGGGTTTTTCTCCTTGACGTTAAAG
6496	ATCGGCATTGATGAGATCCG
6497	cccggcatccgcttacagacaagctgtgaccgtctccgggaCGGCTTCTAATCCGTAC

6498	CGAAGCAAAAATTACGGCTCCTCGCTGCAgatccTCAGTATCAGTACGGATTAGA AGCCG
6499	GCTGAAATATAATTTTCTGTACGCTGCCTTGTGTTTTCTTCC
6515	AAGGCTTTGCAGGTACTG
6516	agctagtcctaggaAAC
6517	GCGCCTAAgAAgAAACGAAAAGTTcctaggcactagctccctgcccctaatacagtactg
6518	tgcgacgtgcggatccccgggttaattaaggcatatgatccgtcgagttc
6519	tttttctcttgaactcgacggatcatatgccttaattaacccggggatcc
6520	aCCCGCTCGGCGGCTTCTAATCCGTAAGTAcacaggcctgatccaatatc
6521	TCTCAGTAATCATGCGGCAG
6522	GTAAGTAacctgctagtgctattcttagctagtgcttaGACCTTGTACAGCTCGTC
6523	ATCACTCTCGGCATGGACGAGCTGTACAAGGTCTaaggcactagctaagaataggc
6524	gaaccggggatgcgacgtgcggatccccgggttaattaagcGACTTGGTCTCTcaacAAC
6525	CTCAATCGGGGTTgttgAGAGACCAAGTCgcttaattaacccggggatcc
6526	ctgctcccggcatccgcttacagacaagctgtgaccgtctcacaggcctgatccaatatc
6531	tcatttctttgatattggatcaggcctgtgTCAGTACGGATTAGAAGC
6532	GAGTGGCAAGGAGTATC
6539	GATTCGTAAACAAGAGGTGC
6552	ACTGAacctgctagtgctattcttagctagtgactaGttaGACCTTGTACAGCTCGTC
6553	GGATCACTCTCGGCATGGACGAGCTGTACAAGGTCTaaCtagtgactagctaagaat ag
6554	ccatcacaAacggcatgatg
6555	AATCGGCCTCTTCGTTAAGC
6556	TTCCCCAGATGAATTGCGTG
6557	gcagccgaacgaccgagcgcgagtcagtgagcaggaAgatccTCAGTATCAGTACG
6558	GTATTAAGCAGCGCGTCCT
6559	GCATATCGACCGGTCGAGGAGAACTTCTAG
6560	CAAAACGACGATCTTCTTAGGGGCAGACATaggggcagacatTAGAATGGTATATC CTTG
6561	CCTCCCACTAATTCTCTGACAACAACGAAG
6562	CAAGGATATACCATTCTAATGTCTGCCCTATGTCTGCCCTAAGAAGATCGTC GTTTTG
6563	TCAACAAGTAATTGGTTGTTTGGCCGAGCGGTCTAAGGCGGCTCGAATTCATC GATagcg
6564	TTTTGTAATTCGTGTCGTTTCTATTATGAATTTCAATTTAttctccttacgcatctgtgc
6565	TCTCAACAAGTAATTGGTTGTTTGGCCGAGCGGTCTAAGGgagtttctgtccactga gc
6566	tgtgaagcgtgacataactaattacatgactcgaggtcggttatccACTCTCTTTTCCCCAC

6567	GGAAGAGGCGCTTCTCAGGTGGGGAAAAGAGAGTggataacgacctcgagtcatgta att
6568	gctcactcaaaggcggttaatacggttatccacagaatcaggcaaattaaagccttcgagc
6569	gcttgagaaggttttgggacgctcgaaggctttaatttgctgattctgtggataaccgt
6570	AGTGATCGTCTAACACCAGC
6575	GGCCTCATCACATAATTATAAACTACAAACCCATTATCAGCAAGTAATGAGTCG ACAACC
6576	aatgctattttgtaatttgaAGCTTTCTGTACATATCGAActagtggatctgatatcacc
6577	CTCCATATTTGCTGGACGTG
6578	attcGCGGGTGCAAACAATG
6579	aggtagtctagtacctcctg
6580	gaacagctaaagggtagtgc
6581	gcaagttcaaggagcgaaag
6582	cgctccacaaacattgctc
6583	ACTTCTCAAGTCCGCCATG
6584	TCTGTAGTTGCCGTCGTC
6585	ACGTAAACGGCCACAAGTTC
6586	AGATCAGCTTCAGGGTCAGC
6587	CAACAAGTAATTGGTTGTTTGCCGAGCGGTCTAAGGCGCCaggccctttcgctctta ag
6588	ggcttgattggagataagtc
6589	TTAGGTCTCGACTCTACTCC
6590	acAACCCCGATTGAGATATAGATTTATTTAAATcaaccggcgtagtatcgaatcgACAG
6591	ATCATGCGTCAATCGTATGTGAATGCTGGTCGCTATACTGCTGTcgattcgatacta acg
6592	taGACCTTGACAGCTCGTC
6593	AGCACTTCTAAGTGAGGGTG
6594	CGCTTATCGCTCCAATTTCC
6595	AAGCATCTGGGAGATCACTG
6596	CCACAACATTAGTCAACTCC
6603	gatacaccagacggaagaac
6604	tgtacctcttcggtcgatg
6605	ccgccttcaagtactttgac
6606	cagaggagcctccgctagatcctccggagggtgatgaattttctatgaggaggtagagg
6607	tccggaggatctagcggag
6608	gcgtgaatgtaagcgtgacataactaattacatgactcgagttagtccagcttcactctc
6624	tcgcgcgctggctgtctactctggagatttccggttgacatgatattactttctgcgcac
6625	gccagatgcgaagttaagtgcgcgaaagtaatatcatgtcaaccggaaatctccagag

6.2. Strains

FK collection number	Genotype
3086	MATalpha ho::LYS2, lys2, ura3, leu2::hisG, trp1::hisG, his3::hisG
3087	MATa ho::LYS2, lys2, ura3, leu2::hisG, trp1::hisG, his3::hisG
3088	MATa/alpha, homozygous for: ho::LYS2, ura3, leu2::hisG, trp1::hisG, his3::hisG, lys2
9381	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, leu2::hisG, trp1::hisG, his3::hisG, ade2-AT49C (ade2-fs1)
9460	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, leu2::hisG, trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, his3::hisG, ade2-AT49C (ade2-fs1)
9469	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, leu2::hisG::pGal1-NLS-JetA::pTEF-NLS-JetB::pTEF-JetC-SV40NLS::LEU2, trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, his3::hisG, ade2-AT49C (ade2-fs1)
9470	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, leu2::hisG::pGal1-NLS-JetA::pTEF-NLS-JetB::pTEF-JetC-SV40NLS::LEU2, trp1::hisG::pGal1-NLS-JetD::TRP1, his3::hisG, ade2-AT49C (ade2-fs1)
9488	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, trp1::hisG, leu2::hisG::pGal1-NLS-JetA::pTEF-NLS-JetB::pTEF-JetC-SV40NLS::LEU2, ade2-AT49C (ade2-fs1)
9489	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, leu2::hisG::pGal1-NLS-JetA::pTEF-NLS-JetB::pTEF-JetC-SV40NLS::LEU2, trp1::hisG::pGal1-NLS-JetD::TRP1, his3::hisG, ade2-AT49C (ade2-fs1)
9490	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, , trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, leu2::hisG::pGal1-NLS-3v5-JetA::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, ade2-AT49C (ade2-fs1)
9526	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, leu2::hisG::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, trp1::hisG, his3::hisG, ade2-AT49C (ade2-fs1)
9527	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, leu2::hisG::pGal1-NLS-3v5-JetA::LEU2, trp1::hisG, his3::hisG, ade2-AT49C (ade2-fs1)
9552	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, , trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, leu2::hisG::pGal1-NLS-3v5-

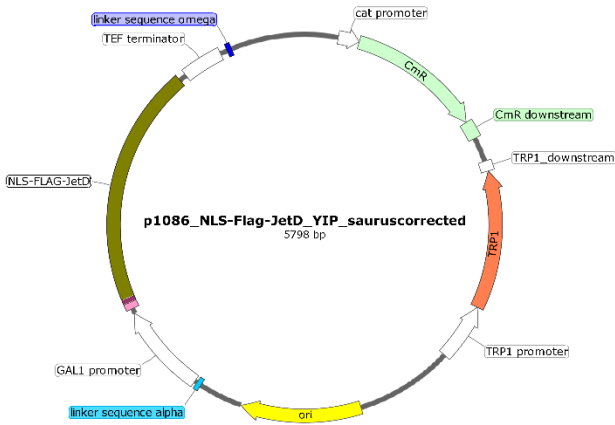
	JetA::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, ade2-AT49C (ade2-fs1) +p1127
9569	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, leu2::hisG::pGal1-NLS-3v5-JetA::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, snq2Δ::KanMX4, pdr5Δ::KanMX4, yor1Δ::NatMX4 YPG+, trp1::hisG +p1127
9570	MATa ho::LYS2, lys2, ura3, leu2::hisG, pdr5Δ::KanMX4, yor1Δ::NatMX4, trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, ade2-AT49C (ade2-fs1)
9571	MATa ho::LYS2, lys2, ura3, trp1::hisG, his3::hisG, leu2::hisG, snq2Δ::KanMX4, pdr5Δ::KanMX4, yor1Δ::NatMX4 ade2-AT49C (ade2-fs1)
9572	MATa ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, leu2::hisG::pGal1-NLS-3v5-JetA::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, snq2Δ::KanMX4, pdr5Δ::KanMX4, yor1Δ::NatMX4, ade2-AT49C (ade2-fs1)
9573	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, leu2::hisG::pGal1-NLS-3v5-JetA::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, snq2Δ::KanMX4, pdr5Δ::KanMX4, yor1Δ::NatMX4, ade2-AT49C (ade2-fs1)
9574	MATa ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, trp1::hisG, leu2::his snq2Δ::KanMX4, pdr5Δ::KanMX4, yor1Δ::NatMX4, ade2-AT49C (ade2-fs1)
9579	MATa ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, leu2::hisG::pGal1-NLS-3v5-JetA::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, snq2Δ::KanMX4, pdr5Δ::KanMX4, yor1Δ::NatMX4, ade2-AT49C (ade2-fs1) +p1127
9580	MATa ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, trp1::hisG, leu2::hisG::pGal1-NLS-3v5-JetA-Venus::LEU2, snq2Δ::KanMX4, pdr5Δ::KanMX4, yor1Δ::NatMX4, ade2-AT49C (ade2-fs1)
9581	MATa ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, leu2::hisG::pGal1-NLS-3v5-JetA::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, snq2Δ::KanMX4, pdr5Δ::KanMX4, yor1Δ::NatMX4, ade2-AT49C (ade2-fs1) +p1138
9582	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, , trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, leu2::hisG::pGal1-NLS-3v5-JetA::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, ade2-AT49C (ade2-fs1) +p1138

9584 (BY)	MATa/MATalpha, his3-Δ1/his3-Δ1; leu2Δ0/leu2Δ0; met15Δ0/met15; lys2/lys2Δ0; ura3Δ0/ura3Δ0, kex2Δ::kanMX
9585	MATa/alpha homozygous for: ho::LYS2, ura3, leu2::hisG, trp1::hisG, his3::hisG, lys2 heterozygous: kex2Δ::kanMX
9587 (BY SK1 HYBRID)	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, , trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, leu2::hisG::pGal1-NLS-3v5-JetA::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, kex2Δ::kanMX
9588	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, , trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, leu2::hisG::pGal1-NLS-3v5-JetA::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, ade2-AT49C (ade2-fs1) +p1138 +p1140
9589	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, trp1::hisG, leu2::hisG::pGal1-NLS-JetA::pTEF-NLS-JetB::pTEF-JetC-SV40NLS::LEU2, ade2-AT49C (ade2-fs1) +p1138 (HIS3) +p1138
9590	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, trp1::hisG, leu2::hisG::pGal1-NLS-JetA::pTEF-NLS-JetB::pTEF-JetC-SV40NLS::LEU2, ade2-AT49C (ade2-fs1) +p1138 +p1140
9591	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, his3::hisG, exo1Δ::KanMX4, trp1::hisG::pGal1-NLS-3V5-JetD::TRP1, leu2::hisG::pGal1-NLS-3v5-JetA::pTEF-NLS-3v5-JetB::pTEF-JetC-3v5-SV40NLS::LEU2, ade2-AT49C (ade2-fs1) +p1138
9595	MATalpha ho::LYS2, lys2, ura3::pGPD1-GAL4(848).ER::URA3, leu2::hisG, trp1::hisG, his3::hisG, ade2-AT49C (ade2-fs1) + p1140

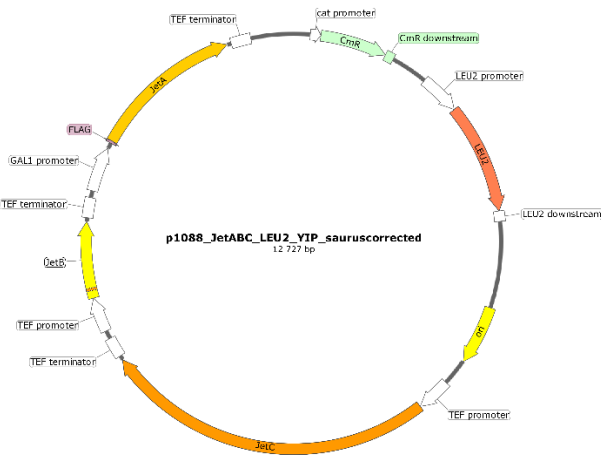
6.3. Plasmids

FK collection number	Map
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1036	

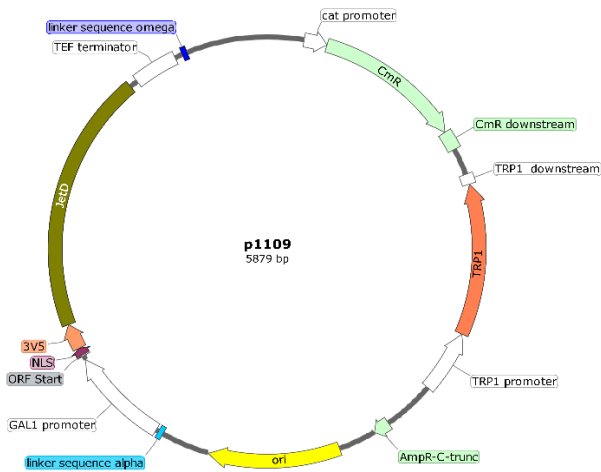
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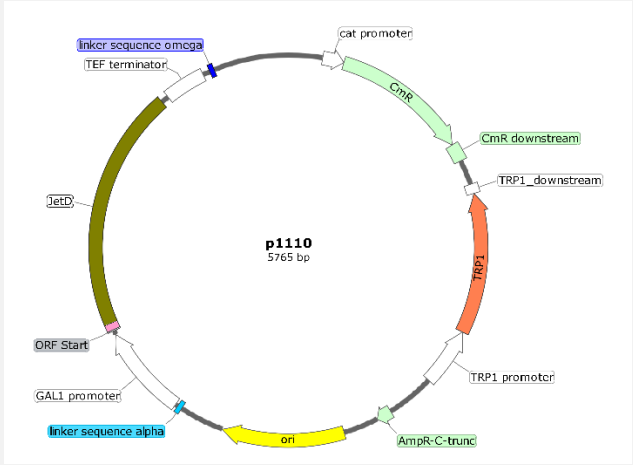
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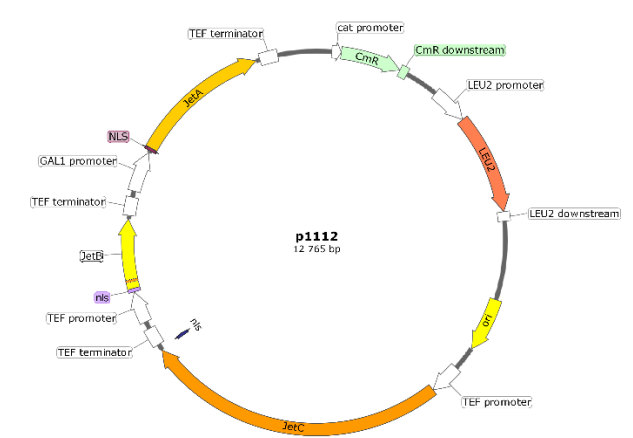
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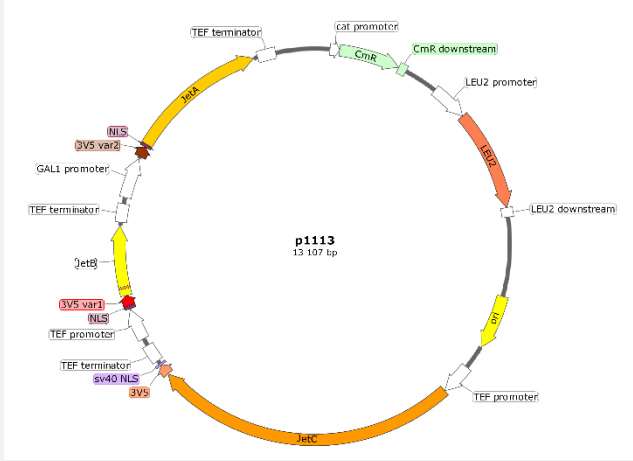
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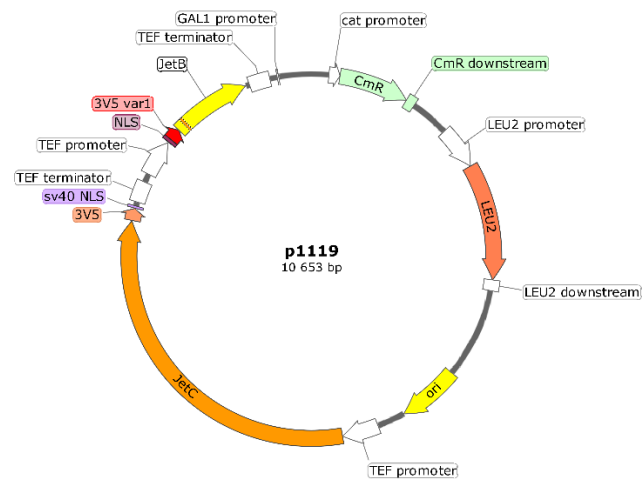
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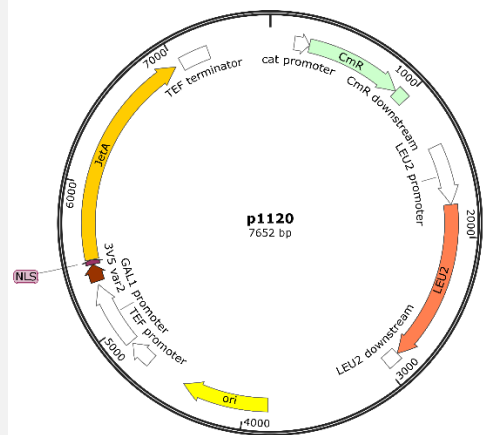
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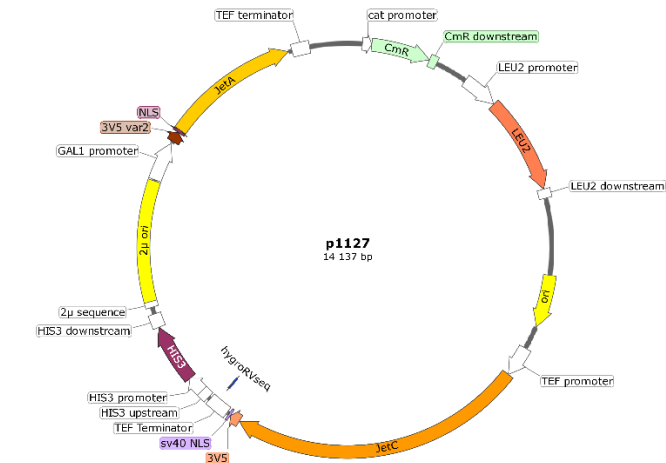
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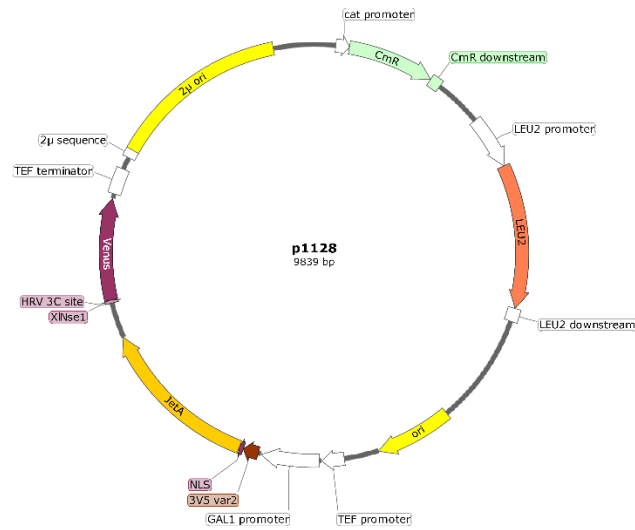
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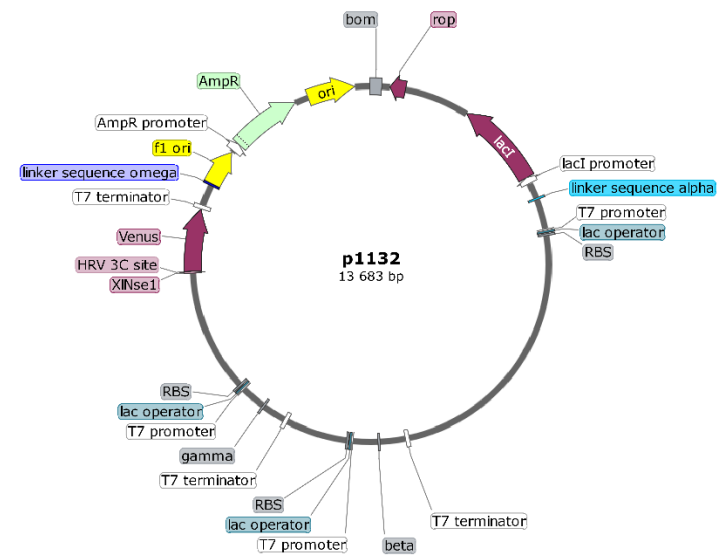
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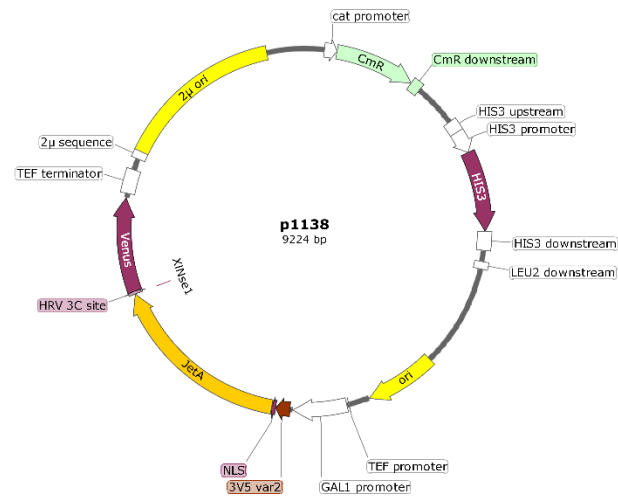
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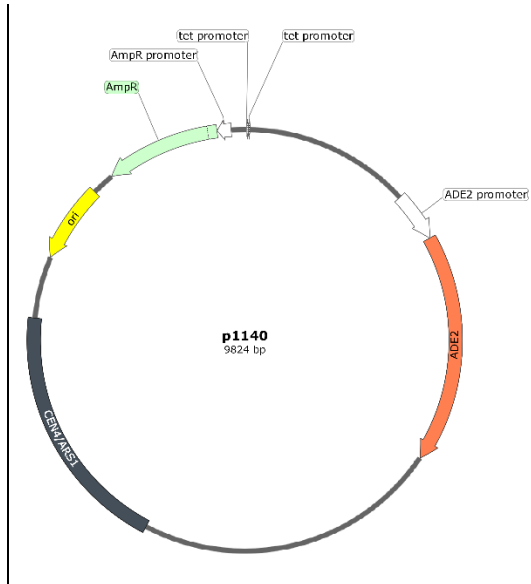
1132



1138



1140



Zusammenfassung

Die Entdeckung von CRISPR hat den immensen Wert von Forschung an bakteriellen Immunsystemen gezeigt. Seither wurden viele neue und diverse Verteidigungssysteme im Pan-Genom der Bakterien gefunden. Besonders einzigartig und vielversprechend unter diesen ist das Anti-Plasmid system WADJET. Es besteht aus vier Proteinen, von denen drei einen SMC-Komplex bilden, der die Topologie der DNA erkennt, wahrscheinlich über Loop Extrusion. Das übrige vierte Protein ist eine Nuklease, welche die DNA schneidet. Zusammen können diese Proteine selektiv zirkuläre DNA, bis zu einem gewissen Größenlimit, identifizieren und schneiden. Ziel dieser Arbeit war es WADJET zum ersten Mal in einem Eukaryoten, der Hefe *S. cerevisiae*, zu erforschen. Wir haben gefunden, dass die Kleisin Komponente, JetA, nicht detektierbar war in ihrer nativen Form, jedoch konnten wir stabile Expression erreichen durch die Fusion des fluoreszenten Tags Venus an den C-Terminus des Proteins. Die Expression der WADJET Proteine war nicht toxisch für die Hefe und sie wurden in großer Zahl im Zellkern gefunden. Wir haben zwei Tests etabliert, um potenzielle Plasmid Restriction durch WADJET nachzuweisen, ein ADE2 Farbsektor-Screen und ein Assay für den direkten Nachweis von Anreicherung linearen Plasmids. Zudem haben wir versucht es an Chromatin-freien Plasmiden zu testen mit Transformation Effizienz als Näherung. Ohne weitere Modifikationen kann WADJET Plasmide in Hefe nicht destabilisieren oder linearisieren. Der Transformations-Assay hat sich als uninformativ herausgestellt, da unerwarteter Weise, unser System für die Induktion mit Estrogen und Gal4.ER Plasmidstabilität, Transformationeffizienz und Zellwachstum beeinflusst.