Rapport de Stage

UNIVERSITÉ DE MONTPELLIER

CRBM
Centre de Recherche en Biologie cellulaire de Montpellier

Functional Relevance of Structural Flexibility in the NuA4 Coactivator Complex

M2 Internship Research Project

MCKEON Conor

M2 Translational Medicinal Chemistry

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Internship duration: 130 days (Jan-July)

Spring, 2022

Summary (300 words)

An important step in gene expression occurs at the level of transcription initiation, which requires the coordinated activities of large, multimeric coactivator complexes that modify histones or remodel nucleosomes, while serving to recruit the general transcription machinery to the promoter. Here we investigated the behaviour of the essential NuA4 coactivator in the fission yeast *Schizosaccharomyces pombe*. Structural evidence shows that in S. pombe, a disordered stretch of 60 amino acids in the Epl1 subunit confers a broad positional flexibility to the Histone Acetyltransferase (HAT) module. To better understand how NuA4 regulates transcription, we analyzed the effects of mutating the essential Epl1 subunit, focusing on this short region linking the HAT module to the rest of the NuA4 complex. To investigate the evolutionary relevance for this striking flexibility, we established a Cre-Lox mediated system to conditionally replace the Epl1 gene with a mutant copy at its endogenous locus, enabling a complete and rapid switch from WT to mutant protein, while preserving its native genomic regulation. Using this system, we characterized the effect of Epl1 linker shortening on yeast growth by replacing the WT Epl1 gene with mutant Epl1 cassettes with different linker deletion lengths. We also designed plasmids such as to deliver a switch from the WT gene to either an identical WT copy or a complete removal of the gene, as positive and negative controls, respectively. We observed dramatic growth defects of yeast cultures upon shortening of the Epl1 linker, although not as severe as observed in cultures which experienced a complete loss of Epl1, suggesting reduced, but not complete loss of functionality of NuA4 upon reducing the flexibility of the linker region. Our findings demonstrate an important role for this unstructured linker region of Epl1, highlighting the potential importance of disordered regions in the function of transcription co-activator complexes.

Introduction

The highly conserved NuA4 coactivator (for Nucleosome Acetyltransferase of H4) has roles in general transcription factor recruitment, histone modification, and DNA double-strand break repair [1,2]. The complex is made up of 13 subunits organized into 5 distinct functional modules [3]. How these modules contribute to transcription and DNA repair remains incompletely characterized, representing an important current topic in epigenetic research. Comparison of unpublished NuA4 cryo-EM structures obtained by collaborators from my host laboratory with published structures X-ray crystallography of the HAT module indicates that the HAT module has a broad positional flexibility, conferred by a stretch of unstructured amino acids in the Epl1 subunit, which form a linker connecting the HAT module to the NuA4 core (Fig. S1A) [4,5]. Previous work showed that linkers connecting different modules of protein complexes can play crucial roles in maintaining cooperative protein–protein interactions [6].

Two studies have that, both *in vitro* and *in vivo*, targeting NuA4 to a specific site within nucleosome arrays generates a broad domain of histone acetylation, spanning up to 8 nucleosomes [7,8]. This finding led us to hypothesize that the positional flexibility of the HAT module relative to the NuA4 core, resulting from flexibility in the Epl1 linker, might be responsible for this long-range catalytic activity of the HAT module at distal nucleosomal targets. We propose that by enabling the HAT module to overcome steric effects and conformational constraints that might otherwise obstruct functional protein-protein interactions, the Epl1 linker confers NuA4 with a potentially important long-range acetylation ability. Here we establish the first functional system in yeast that allows the conditional replacement of a WT allele with a mutant copy at its endogenous locus, preserving its native genomic regulation. Using this system, we induced a permanent genomic switch from WT to mutant Epl1 proteins in which the linker region is shortened and demonstrated a progressive proliferation defect, highlighting the important role of the unstructured Epl1 linker region in NuA4 activity.

Specific Aims and objectives

My goal was to implement and validate the “flip excision” (FLEx) conditional mutant system in *S. pombe* and use it to gain a better understanding of the essential role of NuA4 in transcription regulation. Initial attempts to implement the FLEx system resulted in expression of both the WT and mutant Epl1 protein in uninduced conditions, due to bidirectional transcription initiation from the neighboring TEF1 promoter of the NAT resistance cassette (Fig. S2A). To overcome this issue, we opted to re-design the system to deliver a clean switch from WT to mutant Epl1 upon induction (Fig. S2B). Furthermore, in order to characterize the subunit composition and catalytic activity of the mutant complex, it was necessary to have strains from which we can purify both the WT and mutant NuA4. For this, I designed and constructed a “tagged” strain, in which a Tandem-Affinity Purification (TAP) tag was fused to the core Vid21-subunit of NuA4. This strain then served as a host for transforming and stably integrating the updated FLEx plasmids. Following implementation and validation of the updated FLEx system, my aim was to characterize the phenotypic effects and transcriptomic perturbations resulting from Epl1 linker shortening, to advance our understanding of the molecular mechanisms by which co-activators epigenetically regulate gene expression.

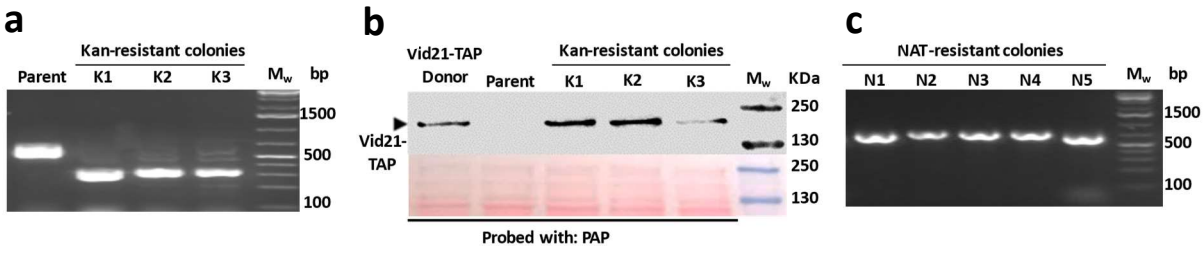

Figure 1: Implementation of the modified FLEx system in a Vid21-TAP background strain. **a** Colony PCR of kanamycin-resistant yeast colonies after transformation with PCR amplified Vid21-TAP from CFP139 (Fig. S3A). **b** Western blot showing Vid21-TAP expression in kanamycin-resistant yeast colonies. **c** Colony PCR of nourseothricin-resistant yeast colonies after FLEx plasmid transformation (Fig. S3B).

Significance, importance, and novelty

Besides well-established histones substrates, the NuA4 complex has been found to acetylate a wide range of non-histone proteins including p53 and c-Myc, with broad-ranging implications in the DNA-damage response, cell-cycle progression, apoptosis, and homeostasis [9-11]. In humans, TIP60, the essential catalytic acetyltransferase subunit of NuA4, is associated with multiple diseases, most notably Alzheimer's disease and cancer [12,13]. Given the diversity of TIP60 functions, it's roles in oncogenesis remain poorly understood, however, consistent with its role in p53 activation, TIP60 appears to function as a tumor suppressor in the majority of cancers studied. [14]. My research aims to reveal how the architecture of the NuA4 complex contributes to its function, and hence reveal a novel regulatory role of NuA4 long-range acetylation. Thus, we hope to provide a valuable insight into the mechanistic elements of NuA4 transcription regulation, which could have significant implications for future research into disease as a result of NuA4 dysregulation.

Methods

Plasmid construction

Five plasmids each carrying a modified Epl1 cassette were designed to test five experimental conditions, hereafter referred to as WT, KO, DCt, SL1, and SL5 (Fig. S1B). These were derived from a plasmid, DHB14, containing a prokaryotic selectable marker conferring ampicillin (AMP) resistance to bacteria, an eukaryotic selectable marker conferring nourseothricin (NAT) resistance to yeast, one LoxP and one LoxM3 site to form the FLEx system upon transformation, and a 600 bp sequence homologous to a genomic region located downstream of the Epl1 gene for stable integration in the *S. pombe* genome (Fig. S3B). The plasmids were linearized before transformation by digestion at an EcoRI restriction site in the homology region, giving two 300bp homology regions at the extremities (Fig. S3B). To construct this template, the four exogenous Epl1 cDNA sequences (three mutants and one WT control) were inserted in an XhoI site following PCR amplification of synthesized DNA fragments (IDT gBlocks). For the negative, KO control, the template plasmid was used as is. Then, Gibson assembly was used to reverse the orientation of the NAT cassette (see the aforementioned observation), inadvertently introducing a second EcoR1 site. To remove the second EcoR1 site and decrease the risk of

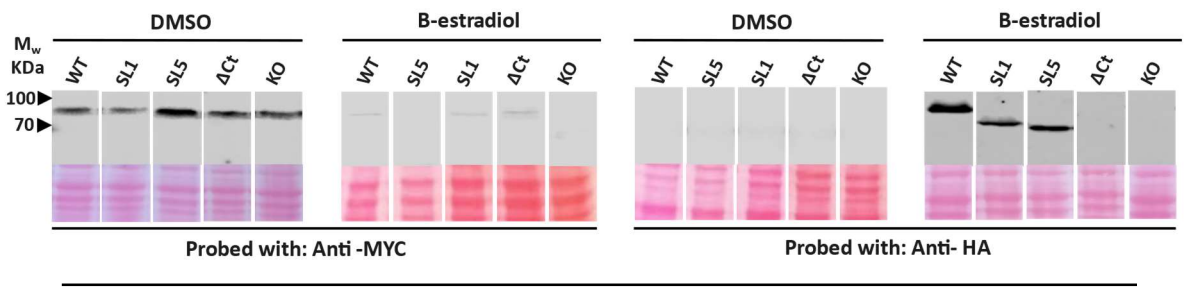

Figure 2: FLEx Switch from MYC-tagged WT Epl1 to HA-tagged mutant Epl1 after 6 hours induction with β-estradiol.

transcriptional readthrough from the NAT cassette in yeast, an ADH1 terminator was subcloned from a plasmid, DHB6, and inserted via Gibson assembly. Digestion with EcoR1 resulted in singly linearized plasmids confirming successful insertion of ADH1. All modifications were validated by Sanger sequencing and restriction fragment analysis.

Strain construction

All *S. pombe* strains used are listed in Supplementary Table 1. Strains were derived from DHP1236, an *S. pombe* strain containing a stably integrated transgene expressing the β-estradiol-inducible CreER fusion protein, in which LoxP and Lox M3 sites were integrated using CRISPR-Cas9 editing at the 5' end of *epl1*, together with a MYC epitope tag in frame with the *epl1* open-reading frame to obtain DHP1700. In the this background, I constructed strain DHP1736 by integrating a Tandem Affinity Purification (TAP) tag in frame with the 3- end of the *vid21* gene, which encodes the core Vid21 subunit of the NuA4 complex, to enable purification of the WT and mutant NuA4 complexes for functional characterization (Fig. S3A). For this, primers were designed to amplify the Vid21-TAP-KAN locus from a previously established strain, CFP139. PCR amplification resulted in a 2900bp amplicon, which was gel-purified before transformation (Machery Nagel, NucleoSpin Gel and PCR Clean-up). DNA transformation into yeast was achieved using a lithium acetate-based protocol as previously described. Each of the three clones obtained was tested for correct genomic integration by PCR and the presence of a band at the predicted size for the Vid-21-TAP fusion was verified by Western Blot (Fig. 1a, 1b).

One of the three resulting strains, DHP1736, was used for the establishment of the 5 FLEx strains by transformation of the linearized plasmids as described in the previous paragraph. This transformation was highly efficient resulting in abundant growth of nourseothricin-resistant colonies. PCR screening confirmed that a large proportion of growing colonies had correct genomic integration of the plasmid (Fig. 1c).

Yeast culture, induction, and growth tests

Liquid starter cultures were inoculated with 3–10-day-old single colonies from solid media plates and grown to saturation (24h) for experiments. Strains were grown in either rich (YES) or minimal (EMM) media at 32 °C to mid-log phase (~0.5 × 10^7 cells per ml). For Cre-lox-mediated recombination, cells were treated with either 1 µM β-estradiol (E2758, Sigma) in DMSO or DMSO

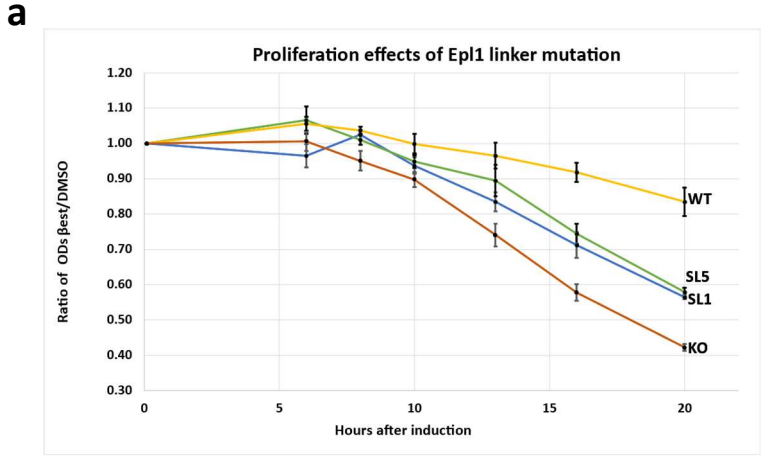

Figure 3: Epl1 linker mutation affects proliferation and colony-forming. **a** Cell-density ratio of induced cultures to uninduced controls following induction **b** Colony-forming ability of uninduced and induced samples after 20 hours of treatment with DMSO or β-estradiol.

alone as a control for the vehicle. Cultures were diluted to keep cells in constant exponential growth.

Protein extraction and Western Blotting

Exponentially growing cells were harvested and flash-frozen. 50mL cultures of exponentially growing cells were homogenized by glass bead beating in a FastPrep (MP Biomedicals). Proteins were extracted using a standard lysis buffer (Workman's Extract Buffer: 40 mM HEPES-NaOH pH 7.4, 350 mM NaCl, 0.1% NP40 and 10% glycerol). WEB was supplemented with protease inhibitors, including complete EDTA-free cocktails tablets (04693132001, Roche), 1 mM PMSF (P7626, Sigma), 1 µg per ml bestatin (B8385, Sigma) and 1 µg per ml pepstatin A (P5318, Sigma). Protein concentrations were measured by the Bradford method and used to load equal amounts of protein across samples for poly-acrylamide gel electrophoresis (PAGE). Ponceau red staining was used to normalize for total protein levels across samples. For Western Blot on nitrocellulose membrane, mouse antibodies (anti-MYC 9b11, anti-HA 16b12) targeting an N-Terminal MYC tag on endogenous Epl1, and an N-terminal HA tag on mutant Epl1 were used to visualize protein size. MYC- and HA-antibody complexes were revealed with anti-mouse horseradish peroxidase. Vid21-TAP was revealed using peroxidase-anti-peroxidase (PAP, Sigma P1291).

Results

The FLEx system induces a complete switch of the endogenous protein.

Colonies screened for Vid21-TAP expression and correct plasmid integration were induced for 6 hours with β-estradiol and DMSO \, before total protein extraction and Western Blot were performed. Anti-MYC antibodies were used to reveal MYC-Epl1, which was found to be expressed in all DMSO-treated samples, as expected (Fig. 2). In contrast, MYC-Epl1 is almost

undetectable in β-estradiol-treated samples, indicating a near-complete depletion of MYC-Epl1 at 6 hours of induction. Upon revelation with anti-HA antibodies, the opposite trend was evident, with HA-Epl1 undetectable in DMSO-treated samples, while β-estradiol-treated samples showed expression of HA-Epl1 at sizes corresponding to the full length "WT" Epl1 protein, and Epl1 with the desired 22 and 44 amino-acid deletions. Absence of any HA-Epl1 signal in the "KO" strain demonstrates an effective gene loss upon induction. The ΔC-terminal mutant protein was not visible by Western Blot, likely reflecting the instability of the truncated protein. ΔC-terminal mutants were excluded from further analyses. The loss of MYC signal and appearance of HA signal upon induction, corresponding to a switch from MYC-Epl1 to HA-Epl1, represents achievement of, to date, the first genetic system enabling the conditional replacement of a protein by another in yeast, while preserving its native regulation.

The disordered Epl1 linker region is important for yeast proliferation

To investigate the importance of the Epl1 linker and thus flexibility of the HAT module, we analyzed the proliferation and colony-forming ability of cultures following β-estradiol induction, relative to DMSO-treated controls. Following induction, we observed a proliferation defect in all cultures in which full-length Epl1 was switched to a mutant copy with a partial or complete deletion of the linker (Fig. 3a). As expected, the Epl1 knockout gave the most severe growth phenotype with cell density of induced cultures less than 50% of that of identical, uninduced cultures at 20 hours post-induction. Interestingly, similar proliferation defects were seen for the strains with 22 and 44 amino-acid Epl1 linker deletions, SL1 and SL5 respectively. In contrast however, SL5 samples showed considerably decreased colony-forming ability compared to SL1 samples after 20 hours of induction (Fig. 3b). This finding led us to hypothesize that the length of the linker region enables the HAT module to pass a "steric threshold" beyond which the positional freedom presumably required to reach histone targets increases significantly.

Discussion

We report to have successfully implemented a leak-free FLEx system in *S. pombe*. We demonstrate a complete and permanent switch at the protein level after 6 hours of induction, enabling temporal analysis of the effect of replacing a gene with a mutant copy, regardless of the essentiality of the target gene. It is the first system in yeast that catalyzes a conditional gene-replacement at an endogenous locus, representing an extremely useful addition to the yeast genetics toolbox. We used this system to demonstrate the effect of shortening the Epl1 linker. Our findings highlight the potential importance of unstructured protein regions, which may often be overlooked in structural studies. This research establishes a basis for further investigations into the function of unstructured regions which are prevalent in subunits of other chromatin-modifying complexes including the conserved SAGA coactivator complex and the Sin3L/Rpd3L histone deacetylation complex.

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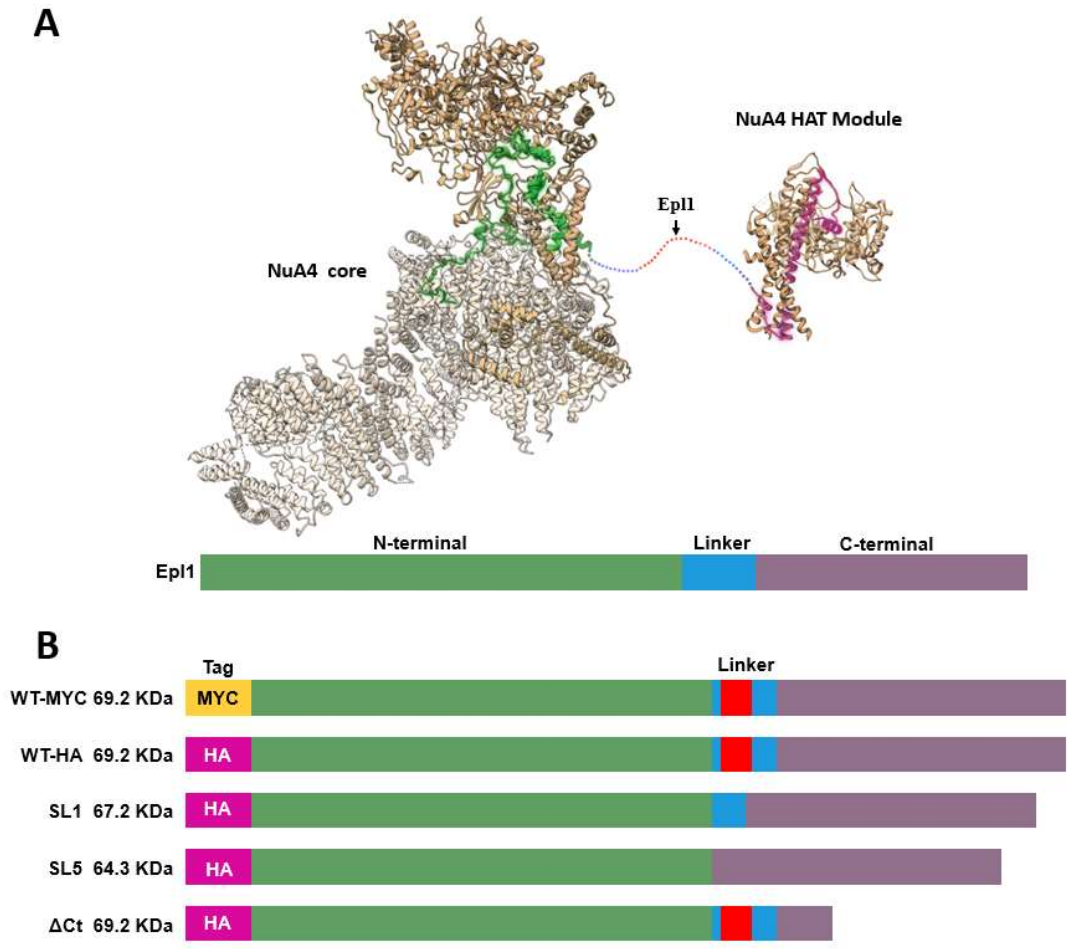
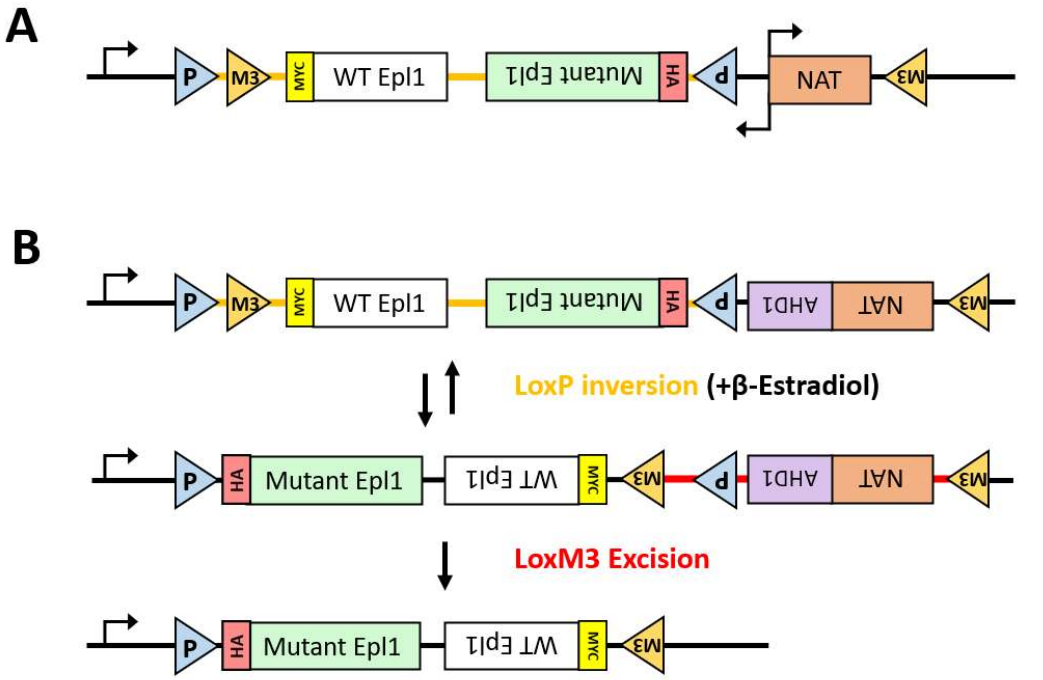
Supplementary Information

Supplementary Table 1: Strains Used

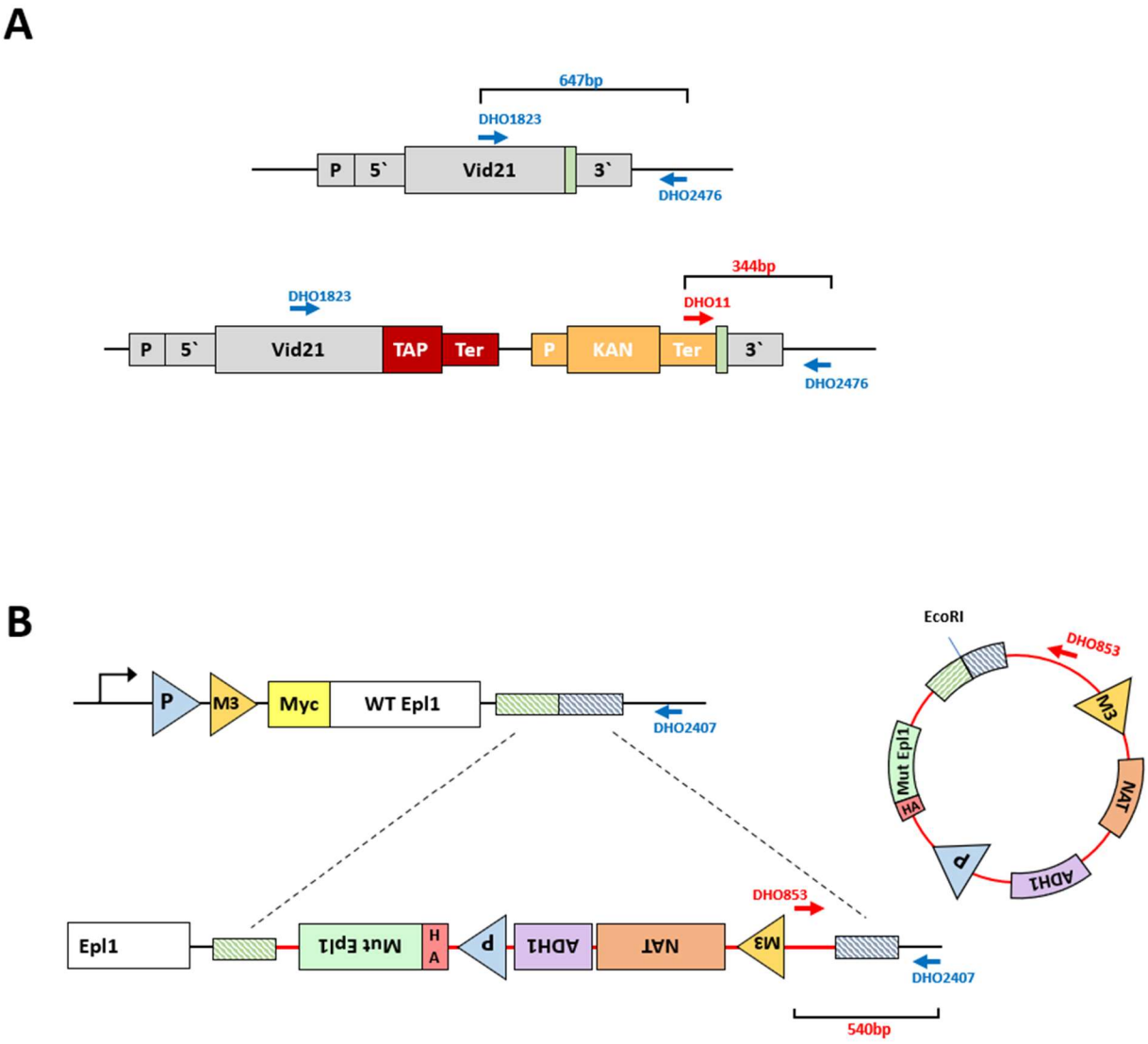
CFP139	Vid21-TAP	Lab strain	CFP123
DHP1700	Cre-ER LoxP-LoxM3-Myc-Epl1	Lab strain	DHP1236
DHP1739	CreER LoxP-LoxM3-Myc-Epl1::FLEx-HA-Epl1-WT	This study	DHP1700
DHP1741	CreER LoxP-LoxM3-Myc-Epl1::FLEx-HA-Epl1-SL1	This study	DHP1700
DHP1743	CreER LoxP-LoxM3-Myc-Epl1::FLEx-HA-Epl1-SL5	This study	DHP1700
DHP1745	CreER LoxP-LoxM3-Myc-Epl1::FLEx-HA-Epl1-KO	This study	DHP1700
DHP1747	CreER LoxP-LoxM3-Myc-Epl1::FLEx-HA-Epl1-ΔCt	This study	DHP1700
DHP1736	Vid21-TAP-KAN CreER LoxP-LoxM3-Myc-Epl1	This study	DHP1700
DHP1749	Vid21-TAP-KAN CreER LoxP-LoxM3-Myc-Epl1::FLEx-HA-Epl1-WT	This study	DHP1736
DHP1751	Vid21-TAP-KAN CreER LoxP-LoxM3-Myc-Epl1::FLEx-HA-Epl1-SL1	This study	DHP1736
DHP1753	Vid21-TAP-KAN CreER LoxP-LoxM3-Myc-Epl1::FLEx-HA-Epl1-SL5	This study	DHP1736
DHP1755	Vid21-TAP-KAN CreER LoxP-LoxM3-Myc-Epl1::FLEx-HA-Epl1-ΔCt	This study	DHP1736
DHP1757	Vid21-TAP-KAN CreER LoxP-LoxM3-Myc-Epl1::FLEx-HA-Epl1-KO	This study	DHP1736



Supplementary Figure 2: **A** – Epl1 links the NuA4 HAT module to the complex core
B – Epl1 mutants used in this analysis



Supplementary Figure 2: **A** – Bidirectional transcription initiation of TEF1 promoter
B – FLEx system schematic



Supplementary Figure 3: A – Vid21-TAP PCR fragment transformation
B – Epl1-FLEx plasmid transformation