Sortase-mediated Ubiquitylation of Histone H2B and its Biological Consequences

by

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ABSTRACT

Regulation of gene expression has been a longstanding puzzle in the understanding of cell biological processes. Histone 2B (H2B) ubiquitylation has been suggested to collaborate in the complex mechanisms that control the activation or silencing of genes. Here we try to explain how we could use the sortagging technique to control ubiquitylation events inside the nuclear envelope. We were able to perform in *Saccharomyces cerevisiae* an *in vivo* SrtA-mediated intramolecular circularization reaction of the NLS-tagged enhanced green fluorescent protein that contains an N-terminal glycine residue and a C-terminal sortagging motif (G-NLS-eGFP-LPETG-myc). However, nuclear fractionation experiments were unable to show efficient nuclear localization of NLS-SrtA and the modified eGFP. We designed and expressed in yeast a sortaggable H2B molecule that could be used for intranuclear sortase-mediated histone ubiquitylation. For the benefit of future SrtA experiments, we produced mouse polyclonal antibodies against *Staphylococcus aureus* and *Streptomyces pyogenes* SrtA. These experiments will help to further the development of intranuclear sortagging reactions in yeast and to apply the technique to perform inducible H2B ubiquitylation.

Thesis Supervisor: Hidde L. Ploegh
Title: Professor of Biology

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Introduction

Regulation of gene expression is achieved by a balance between activation and silencing of DNA regions that result in the required mix of signals to control cellular physiology. Reversible histone modifications play a central role in this regulation. The addition and removal of methyl, acetyl, phosphoryl, sumoyl, and ubiquityl groups, together establish a complex histone code that enables tight regulation of chromatin domains (Bannister and Kouzarides, 2011). Different modifications, alone or in combination, produce different effects on the final state of chromatin structure by affecting histone-protein or histone-DNA interactions.

Histone ubiquitylation is one modification that affects gene expression, but, the mode of regulating this modification and its consequences remain to be explored in detail. H2A was the first ubiquitylated protein to be characterized in 1975 (Goldknopf et al., 1975) and, since then all histones have been shown to exist with this modification. Models based on recent studies suggest that histone H2B ubiquitylation may affect the physical interactions of DNA with the nucleosome, mediate histone crosstalk, or tag H2B for degradation by the ubiquitin-proteasome system (UPS) (Geng and Tansey, 2008; Fierz et al., 2011).

Ubiquitin is a 76 amino acid protein that is processed from its precursor by a deubiquitylating enzyme (DUB) and becomes a substrate for the E1-E2-E3 cascade(Komander, 2009). Ub is activated by an ATP-dependent E1 (Ub-activating) enzyme and transferred to one of approximately 20 E2 (Ub-conjugating) enzymes. This Ub-E2 complex is recognized by one of hundreds E3 (Ub-ligase) enzymes, which facilitate the transfer of Ub to a lysine, less frequently Ser, Thr or Cys residue

in the substrate. Substrates may be subjected to mono- or polyubiquitylation by the subsequent attachment of Ub to Lysine 6 (K6), K11, K29, K48, or K63 onto the growing polyubiquitin chain, resulting in different functionality(Komander, 2009). For example, K48 and K11-linked polyubiquitin chains target substrates for proteasomal degradation at higher rates than observed with other types of linkages.

H2B is both mono- and polyubiquitylated, although the effects of each are not fully understood (Geng and Tansey, 2008). In yeast, H2B ubiquitylation at K123 is mediated by the Brel-Rad6 E2/E3 complex, which requires the polymerase II-associated factor (Paf) complex for activity. The Rtfl subunit of the Paf complex allows the Brel-Rad6 complex to associate with RNA polymerase II (Fleming et al., 2008). Substitution of K123 with arginine (K123R) completely abolishes H2B-ub and confers defects in mitotic cell growth and meiosis (Robzyk et al., 2000). Recent studies in human MCF7 cells have linked decreased H2B-ub with defects in transcriptional elongation upon knockdown of RNF20, the human homolog for Brel (Prenzel et al., 2011). This suggests that H2B-ub has effects on DNA interactions with the nucleosome complex, a requirement for efficient transcription elongation by RNA polymerase II, and that this modification may have similar roles in DNA dynamics in other eukaryotes.

Protein degradation via the UPS requires the activation of ubiquitin-dependent chaperones in charge of disassembling protein complexes and unfolding proteins prior to degradation. One such chaperone is the valosin-containing protein (VCP), also called p97 in mammals, Cdc48p in yeast, which is highly conserved among all eukaryotes(Yamanaka et al., 2011). The barrel-shaped hexameric AAA+

ATPase uses chemical energy from ATP hydrolysis to mechanically change the conformation of its ubiquitylated substrates. VCP/p97/Cdc48p is involved in a broad range of biological processes: proteolysis, ER-associated protein degradation (ERAD), chromosome condensation, DNA damage response, and DNA replication, among others. The general role of Cdc48p is to separate substrates from their interacting partners by recognizing them with the help of substrate-recruiting cofactors, and process them using substrate-processing cofactors. In this manner Cdc48p determines their fate by either targeting them for degradation via polyubiquitylation, or release them as stable monoubiquitylated or deubiquitylated proteins (Stolz et al., 2011).

The effects of H2B ubiquitylation and Cdc48p activity on nuclear processes have been found to be similar like, for example, in the DNA damage response and DNA replication. It has been suggested that H2B polyubiquitylation may render it a substrate for Cdc48p, not so much as a target for degradation, but rather as a way to disassemble nucleosomes for histone exchange (Deichsel et al., 2009). Recent studies have also linked p97 activity with degradation of cdc10-dependent transcript 1 (CDT1), which serves as a licensing factor for replication initiation as part of the prereplication complex (Franz et al., 2011; Raman et al., 2011). Degradation of CDT1 upon DNA replication initiation prevents re-replication of DNA to ensure accurate DNA duplication. The same mechanism is used during the DNA damage response to prevent DNA replication in the presence of broken double-stranded DNA. Double-stranded DNA breaks (DSB) induce H2B ubiquitylation and RNF20 colocalization to homologous recombination repair sites (Nakamura et al., 2011).

DSB repair requires chromatin remodeling and rearrangement of H2A/H2B pairs to make DNA accessible to the repair machinery. It is not known whether a connection exists between H2B ubiquitylation and Cdc48p segregase activity in relationship to transcriptional activation, DNA replication or DNA damage response. Also, it is not clear whether these processes rely on local disruption of protein complexes or proteosomal degradation by the concerted effects of Cdc48p activity and H2B-ub.

In order to address these questions it would be of great help to be able to control the state of H2B ubiquitylation in an inducible manner. We could then observe the consequences of this modification and do so by specifically targeting H2B without perturbing other UPS-dependent processes. Sortase is becoming a valuable tool in molecular biology for targeted protein modifications (Popp and Ploegh, 2011). Its specificity allows the attachment of a variety of functional groups of choice to soluble and surface proteins. Different applications range from protein labeling for microscopic observations to *in vitro* protein engineering. SrtA catalyzes a transpeptidation reaction with a mechanism similar to that of cysteine proteases (Huang et al., 2003)(Figure 1). A substrate with the LPXTG sorting motif is cleaved between the threonine and glycine residues producing an acyl-enzyme intermediate. This can be subsequently resolved by linkage of the threonine in the substrate to the amino group of a nucleophile that contains an oligoglycine on its amino terminus. In this thesis we will describe our approach to make nuclear sortagging a reality and how this method can be used for the study of the effects of H2B ubiquitylation on gene expression.

Results

Nuclear circularization of GGG-NLS-eGFP-myc

Nuclear fractionation experiments were performed in order to analyze the localization of the circularization reaction by sortase of G-NLS-eGFP-LPETG-myc (Figure 2). This allowed us to evaluate the colocalization of the enzyme and substrate within the nuclear compartment. We were also interested in determining the level at which the reaction is occurring within the nucleus and outside the nuclear envelope.

The CIT1 promoter controlled G-NLS-eGFP-LPETG-myc expression and GAL1 promoter controlled NLS-SrtA-HA expression. The full length linear G-NLS-eGFP-LPETG-myc and G-eGFP-LPETG-myc products are observed as ~ 31 kDa and ~30 kDa polypeptide, respectively. An additional polypeptide is observed at 25 kD, possibly due to non-specific binding of the α-GFP antibody. Two hours after galactose induction of NLS-SrtA-HA expression, we observed the appearance of a faster-migrating 26 kD band that matches results observed in cytosolic and ER eGFP circularization experiments(Strijbis et al., 2012). However, the same band was observed when the cytosolic eGFP was expressed, suggesting that part of the reaction has occurred outside the nuclear envelope, a not entirely unexpected result. Hydrolysis products were also observed at a lower molecular weight than circularized eGFP, possibly due to the presence of ε-amines from lysine side chains close to the sortase recognition sequence that could act as nucleophiles. The nuclear fractions show a low signal of G-NLS-eGFP-LPETG-myc and NLS-SrtA-HA when

compared to the levels observed on the cytosolic fractions. This could be due to inefficient import through the nuclear envelope or a non-functional NLS.

The signal from the myc tag located C-terminal to the LPETG on G-NLS-eGFP-LPETG-myc and G-eGFP-LPETG-myc serves the purpose of discriminating between the linear and circularized eGFP. After the formation of the thioacyl intermediate between sortase and the substrate, the C-terminal epitope tag will be released, resulting in the loss of the myc tag signal. After induction of NLS-SrtA-HA expression with galactose as detected by α-HA antibody, the signal of α-myc antibody decreased in intensity. None of the lower molecular weight species in the cytosolic fraction detected with α-GFP antibody show a signal with the α-myc antibody, suggesting that the lower bands have lost the myc epitope tag after cleavage by NLS-SrtA-HA. Nevertheless, the nuclear fraction failed to indicate the presence of the myc tag signal on G-NLS-eGFP-LPETG-myc, casting doubt on the effectiveness of the NLS in localizing the proteins to the nucleus.

SrtASp-mediated H2B ubiquitylation

Haploid S. cerevisiae contains two loci for the expression of H2B, HTB1 on chromosome IV and HTB2 on chromosome II. The LPETGGG-3HA sequence was inserted at the 3' end of the HTB1 gene, resulting in the expression of H2B-LPETGGG-3HA together with endogenous H2B from the HTB2 locus. Positive transformants were selected by G418 resistance and confirmed by PCR.

Western blots of cell lysates containing the modification show a band at ~ 22 kD as detected by α-H2B and α-HA antibodies in contrast to the wild-type H2B band

of ~15 kD (Figure 3). Since H2B expression also originates from the HTB2 loci, there is a 50% decrease in α-H2B signal compared to wild-type.

The production of SrtA antibodies facilitated the identification of the enzyme on yeast lysates without the need of an epitope tag. BALB/c mice were immunized with a mix of *S. aureus* and *S. pyogenes* SrtA purified from *E. coli* as used for *in vitro* sortagging reactions. ELISA showed high reactivity of immune mouse sera with both species of SrtA. Western blot analysis of yeast lysates after galactose induction show that a 1:3000 dilution of serum allows the observation of NLS-SrtA-HA in yeast lysates.

Discussion

We have described a method for targeting sortagging reactions within the nuclear membrane by localizing the enzyme NLS-SrtA-HA and G-NLS-eGFP-LPETG-myc as a substrate with an internal nucleophile to achieve compartment-specific circularization of proteins. We have also explained our approach to achieve SrtA-mediated ubiquitylation of LPETG-tagged H2B by the use of nuclear SrtA and ubiquitin as a nucleophile. Unlike *in vitro* reactions, intracellular sortagging relies on the cellular conditions present in the compartment to which the enzyme, substrate, and nucleophile have been targeted. This makes optimization of the reaction difficult to control, relying mostly on the fact that all three molecules must have access to the precise location within the cell for the acyl-enzyme intermediate to be resolved by the desired nucleophile.

Due to low concentrations of NLS-SrtA-HA and G-NLS-eGFP-LPETG-myc within the nuclear envelope, as determined by nuclear fractionation experiments, we

could not determine with confidence that nuclear sortagging can occur in the conditions present within the nucleus. Previous studies have shown that in some cases there is low import rate of GFP fusions when the SV40 T antigen NLS is used (Chatterjee et al., 1997), therefore, it is important to optimize the transport of SrtA into the nucleus by the use of different nuclear localization signals that could improve passage through the nuclear pore complex. We used CIT1 and GAL1 promoters, which produce high amounts of protein constitutively and after galactose induction, respectively. The use of less active promoters, like CYC1 and GALS, may improve the efficiency at which the NLS-containing molecules are imported into the nucleus (D Mumberg, 1994; Mumberg et al., 1995).

Another advantage of efficient nuclear transport is that the availability of the desired nucleophile close to the acyl-enzyme intermediate will decrease undesired nucleophiles to resolve the enzyme-substrate intermediate. Hydrolysis or ε-amines on lysine side-chains close to the SrtA binding site could decrease the concentration of intact substrate necessary to obtain the desired final product (Ton-That et al., 2000; Möhlmann et al., 2011). Eliminating these alternate reactions will optimize the sortagging reaction and increase the yields of relevant products.

Methods

Plasmids

All plasmids constructs used in this work are listed on Table 1 and the cloning scheme on Figure 4. The NLS-SrtA-HA sequence was obtained from pKS78 (Strijbis et al., 2012) using primers 5'- GGTCTAGAATGGGACCAAAAAAGAA

GAGAAAGGTATCCAGTGTCTTGCAAGCAAAAT-3' and 5'-GGCTCGAGCTACTCGCTTGCGGCCC-3'. The G-NLS-eGFP-LPETG-myc sequence was obtained from pKS91 (Strijbis et al., 2012) using primers 5'-GGTCTAGAATGGGTCCAAAAAAGAAGAGAAAGGTAACCATTCCCTTA GGATCCGTGA-3' and 5'- CCCTCGAGCTAATC CTCGCTTGCGGCCCC-3' Chromosomal integrations were performed using the system of shuttle vectors described by (Sikorski and Hieter, 1989) (Figure 4). Insertion of the LPETGGG signal and HA tag into the 3' end of the HTB1 site was performed as described by (Longtine et al., 1998) with the modifications described below. The LPETGGG signal was inserted into the pFA6a-3HA-KanMX6 vector at the PacI site by an annealed pair of synthetic oligonucleotides with sequences 5'-TAATCTTCCTGAAACTGGTGGTGGTTTAAT-3' and 5'-TAAACCA CCACCAGTTTCAGGAAGATTAAT-3'. The integration PCR fragment for the HTB1 3' end was amplified using primer F2 with sequence 5'-TGGCTAAGCATGCTGTCTCTGAAGGTACTAGAGCTGTTACCAAGTACT CTTCCTCTACTCAAGCACGGATCCCCGGGTTAATTAA-3' and primer R1 with sequence 5'-CGAGAACACAATTTTACAACCGAGTAAATATCTTAT AATCATTAAAATTATTAAACTATTGAATTCGAGCTCGTTTAAAC-3'. The nuclear localization signal (NLS) used was from the SV 40 T antigen as described in (Shulga et al., 1996). Constitutive protein expression was controlled by the citrate synthase 1 (CIT1) promoter using the 600-bp sequence upstream of the translation start site of the gene, while inducible expression was controlled by the GAL1 promoter (pGAL).

Strains and Culture Conditions

Yeast cultures were grown on YPDA media (1% Bacto yeast extract, 2% Bacto peptone, 2% dextrose, and 0.002% adenine sulfate) at 30°C. Selections of URA3, LEU2, and HIS3 strains were performed by growth in synthetic complete (SC) dropout media (0.67% yeast nitrogen base without amino acids, 2% agar) with the addition of supplements as described in (Green). G418-resistant strains were grown in SC dropout media without ammonium sulfate and 500 mg/ml of G418 (Invitrogen). All yeast strains (listed on Table 2) were produced by transformation of W303 using a linearized fragment of the plasmids listed on Table 1, with the exception of JDY26 that was transformed with the insertion of a PCR fragment as described above.

Protein Expression and Western Blot

Galactose inductions were performed by inoculation of 5 ml of YPDA from the corresponding selection agar plate and grown overnight at 30°C. Each overnight culture was diluted to an OD_{600} of 0.20 in 20 ml and incubated for 1 hour at 30°C. A 10 OD_{600} units sample was gathered for the 0 hour time point, then cultures were induced with 2% galactose and incubated at 30°C. A 10 OD_{600} units sample was taken at time point 1, 2, 3, or 4 hours. Cells resuspended with PBS buffer containing 0.5 mM phenylmethylsulfonyl fluoride and cOmplete protease inhibitors (ROCHE) were lysed by vortex at 4°C using glass-beads. Lysates were resolved by Tris/Glycine SDS-PAGE and transferred to nitrocellulose using the SD trans-blot (BIO-RAD). GGG-NLS-eGFP-myc was detected using a α-GFP-HRP antibody to a 1:200 dilution and α-myc antibody to a 1:5000 dilution. NLS-SrtASp-HA and H2B-

LPETGGG-3HA was detected using a 1:3000 dilution of α-HA antibody. Wild-type H2B was detected using a 1:5000 dilution of α-H2B. Phosphoglycerate kinase (PGK) was used as a loading control and detected using a 1:5000 dilution of α-PGK.

SrtASp and SrtASa Polyclonal Antibody Production and ELISA

BALB/c mice were immunized with a mixture of 40 μg of each SrtASp and SrtASa on day 0. On day 11, the five mice were boosted with a mixture of 10 μg of each SrtASp and SrtASa. This was repeated on day 18 and 25. Bleedings were performed on day 18 and day 28 to test for the presence of polyclonal antibodies. To quantify the titer of antibody on mouse serum, Enzyme-linked Immunosorbent Assay (ELISA) was performed as described on (Hornbeck et al., 1991) with the modifications detailed below. Protein binding was performed using 20 μg/ml in of SrtASp or SrtASa on 0.1 M CO_3^{-2}/HCO_3^- buffer (pH 9.5) in a 96-well plate format. A maximum concentration of a 1:50 dilution of serum was used for the dilution gradient. A 1:5000 dilution of secondary α-IgG HRP-conjugated antibody was used. After the addition of the NPP substrate, the reaction was stopped with 50 μl of 0.5 M sulfuric acid.

Nuclear Fractionation

All yeast nuclear fractionations were performed as described in (Kizer et al., 2006). The final nuclei pellet was washed 3 times with 500 μl and resuspended in 200 μl of NP buffer. Fractions were resolved via Tris/Glycine SDS-PAGE using a volume ratio of cytosol to nucleus of 6:1 and then analyzed by Western blot. α-H2B was used as a nuclear marker and α-PGK as a cytosolic marker.

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Figure Legends

Figure 1

Scheme of sortase-mediated ubiquitylation reaction of H2B inside the nuclear envelope.

Figure 2

Nuclear fractionation of G-NLS-eGFP-LPETG circularization reactions after galactose induction of NLS-SrtA-HA in *S. cerevisiae*. All yeast nuclear fractionations were performed as described in (Kizer et al., 2006). The final nuclei pellet was washed 3 times with 500 µl and resuspended in 200 µl of NP buffer. Fractions were resolved via Tris/Glycine SDS-PAGE using a volume ratio of cytosol to nucleus of 6:1 and then analyzed by Western blot. α-H2B was used as a nuclear marker and α-PGK as a cytosolic marker.

Figure 3

Western blot of H2B-LPETGGG-3HA expression. W303 (WT) as wild-type does not express the LPETGGG-tagged version of H2B. Two colonies were selected after transformation with an insertion PCR fragment. Colony 2 shows expression of H2B-LPETGGG-3HA.

Figure 4

Cloning scheme. (A) Chromosomal integrations were performed using the system of shuttle vectors described by (Sikorski and Hieter, 1989). GAL1 and CIT1 promoters were used for NLS-SrtA-HA and eGFP, respectively. (B) The LPETGGG signal was inserted into the pFA6a-3HA-KanMX6 vector at the PacI site. The PCR fragment will be inserted by homologous recombination into the 3'-end of the HTB1 gene before the stop codon and 85 bp after.

Figures

Figure 1

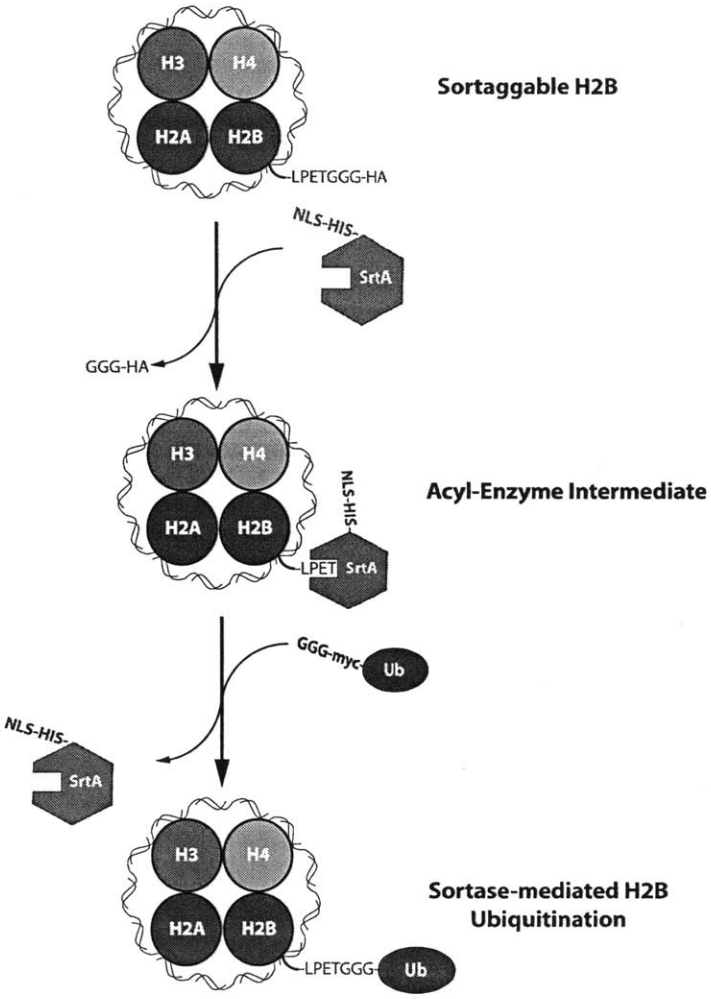
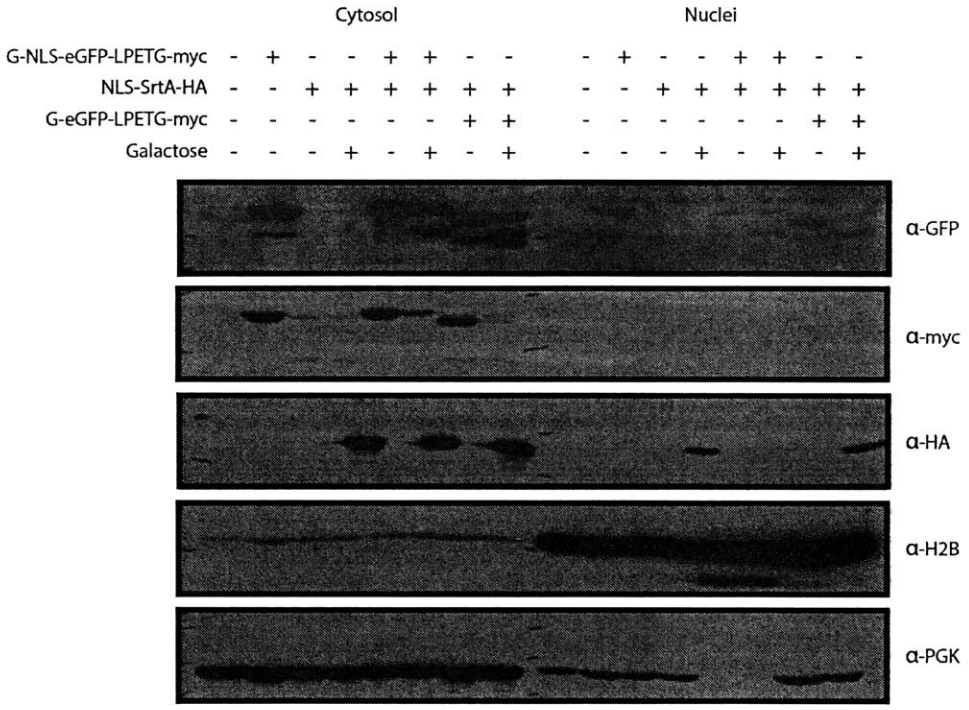


Figure 2


Cytosol
Nuclei
G-NLS-eGFP-LPETG-myc - + - - + + - - - + - - + + - -
NLS-SrtA-HA - - + + + + + + - - + + + + + +
G-eGFP-LPETG-myc - - - - - - + + - - - - - - + +
Galactose - - - + - + - + - - - + - + - +
α-GFP
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α-H2B
α-PGK

Figure 3

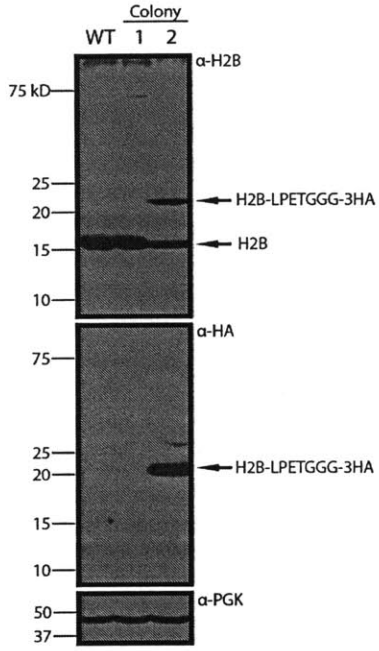
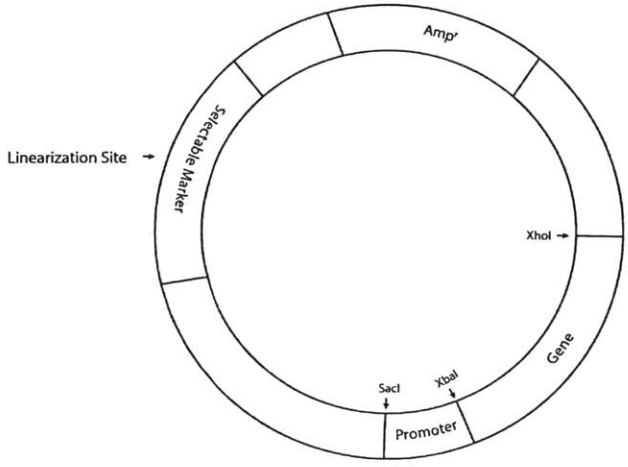
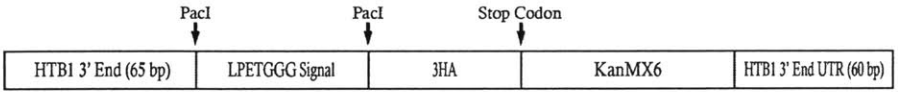


Figure 4

A



B



Tables

Table 1. List of Plasmids

Plasmid	Promoter	Gene	Selection	Reference
pJD10	-	pFA6a-LPETGG-HA	KanMX6	This thesis.
pJD13	CIT1	G-NLS-eGFP-LPETG-myc	LEU2	This thesis.
pJD17	GAL1	NLS-SrtA-HA	HIS3	This thesis.
pKS92	CIT1	G-eGFP-LPETG-myc	URA3	Strijbis et al. 2012

Table 2. List of Strains

Strain	Genotype
JDY11	P_{CIT1}-G-NLS-eGFP-LPETG-myc::LEU2
JDY19	P_{GAL1}-NLS-SrtA-HA::HIS3
JDY20	P_{CIT1}-G-NLS-eGFP-LPETG-myc::LEU2 P_{GAL1}-NLS-SrtA-HA::HIS3
JDY25	P_{CIT1}-G-eGFP-LPETG-myc::URA3
JDY26	$Htb1^{LPETGGG\text{-}3HA}$::Htb1