# CHAPTER 3

## TO DETERMINE THE POTENTIAL OF DEGRADATION DETERMINANT SIGNALS (DEGRONS) OF ORNITHINE DECARBOXYLASE FOR TARGETED PROTEIN DEGRADATION IN *S.CEREVISIAE*

# Determination of the potential of degradation determinant signals (degrons) of ornithine decarboxylase for targeted protein degradation in *S.cerevisiae*

## 3.1 Introduction

As mentioned earlier in Chapter 1, researchers have used full length ODC sequence to achieve targeted protein degradation (Matsuzawa et al., 2005). The major side effects with full length ODC after over expression are: (i) it has massive size and (ii) enzymatic activity, resulting in increased polyamine level in the cells. The present study focuses on engineering the degrons of ODC as efficient vehicle for targeted protein degradation, keeping the degrons intact and pruning ODC to minimal size.

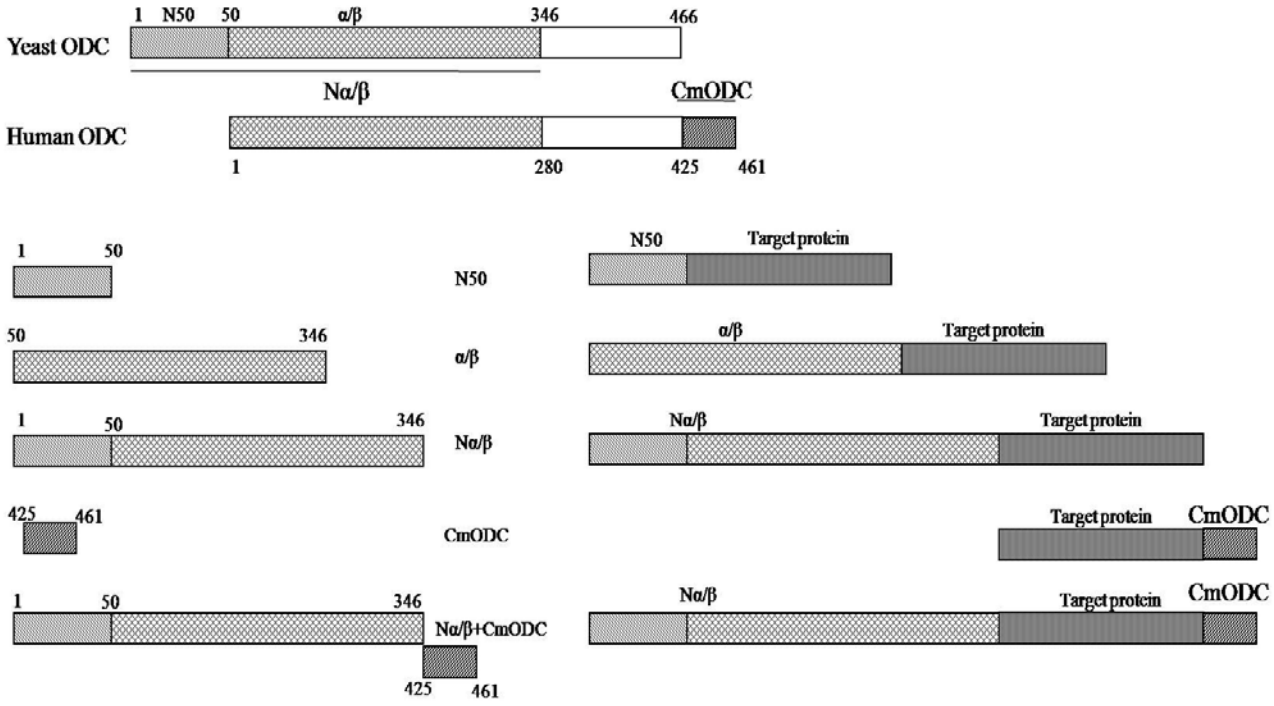


**Figure 3.1 Comparison of yeast and mouse ODC and pictorial representation of yODC and mODC degrons and their chimeric protein**

In this study, we have selected five peptide sequences representing the degrons of yODC and mODC. The degrons are (i) N-terminal first 50 residues of yODC (N50), (ii) the α/β domain of yODC, (iii) the combination of two sequences N50 + α/β (Nα/β), (iv) last 37 residues from mouse ODC (CmODC) and (v) combination of Nα/β and CmODC. The degrons of ODCs have been tagged to reporter protein at N-terminal, C-terminal and both N & C terminal to check their degradation potential to act as signals for targeted protein degradation (Fig. 3.1).

## 3.2 Materials and methods

### 3.2.1 Strains and plasmids

The strains of *Saccharomyces cerevisiae* used in the present work are Y05034 (ΔODC) derivative of BY4741 and Y651 (ΔOAz1). Details of strains and plasmids used in this study are described in Table 3.1.

**Table 3.1 Strains and plasmids used in this study**

| Strains and Plasmids | Details |
|---|---|
| *S. cerevisiae* Y05034* | *MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 spe1*::*KANMX* |
| *S. cerevisiae* Y651* | Mat**a** *his3 leu2 lys2 ura3 oaz1*Δ::*KANMX* |
| pUG35 plasmid** | Yeast expression vector expressing *yEGFP* gene under *ADH* promoter. Multiple cloning site is present upstream to *yEGFP* gene |
| pUG46 plasmid** | Yeast expression vector expressing *yEGFP* gene under *ADH* promoter. Multiple cloning site is present downstream to *yEGFP* gene |
| pUb23 plasmid[#] | Yeast expression vector expressing *Ub-lacZ* gene under *GAL10* promoter. |
| pUG-N50-yEGFP | Expressing N50-yEGFP chimeric protein |
| pUG-α/β-yEGFP | Expressing α/β-yEGFP chimeric protein |
| pUG- Nα/β- yEGFP | Expressing Nα/β-yEGFP chimeric protein |

| | |
|---|---|
| pUG-N50-Ura3-yEGFP | Expressing N50-Ura3-yEGFP chimeric protein |
| pUG- Nα/β- Ura3-yEGFP | Expressing Nα/β-Ura3-yEGFP chimeric protein |
| pUb23-N50-β-galactosidase[@] | Expressing N50- β-galactosidase chimeric protein |
| pUb23- Nα/β- β-galactosidase[@] | Expressing Nα/β- β-galactosidase chimeric protein |
| pUG-yEGFP-CmODC | Expressing yEGFP-CmODC chimeric protein |
| pUG- Nα/β- yEGFP-CmODC | Expressing Nα/β-yEGFP-CmODC chimeric protein |

* Prof. Herbert Tabor, National Institutes of Health provided *S. cerevisiae* strains

** Jeffrey Gerst, The Weizmann Institute of Science provided plasmids pUG35 and pUG46

# Professor Daniel Finley, Harvard Medical School provided pUb23 plasmid

@ Chimeric plasmids were constructed in our laboratory (Sapnali Kulkarni thesis 2012)

### 3.2.2 Cultural conditions

*Saccharomyces cerevisiae* strains Y05034 and Y651 expressing target protein and its fusion proteins with degrons of ODCs were grown in fresh synthetic dextrose (SD) medium under selection pressure. ODC gene (*spe1*) was deleted in Y05034 strain. It cannot grow in the absence of polyamines. So, this strain was supplied with 100 µM putrescine in culture medium. The strains Y05034 of *S. cerevisiae* can tolerate 10 mM putrescine (Teixeira et al.), though one of the reports suggests that the strain can tolerate up to 80 mM putrescine without suffering any negative effects (Tachihara et al., 2005).

In proteasome inhibition study, *S. cerevisiae* Y05034 strain was supplied with 100 µM putrescine in culture medium, with and without proteasome inhibitor MG132 (50µM).

The concentration of putrescine used in the culture medium was varied from 1µM to 1000 µM in the experiments where the effect of antizyme on degradation was studied. Cultures of Y651 expressing target protein and its fusion protein with degrons were also used for studying the effect of antizyme on degron mediated degradation.

The cultures of Y05034 and Y651 strains of *S. cerevisiae* expressing the chimeric proteins were incubated with and without cycloheximide to find the reason behind differences in fluorescence intensities of cultures expressing the various fusions of degrons. The cultures of Y05034 were grown in 1000 µM putrescine with 4mg/ml cycloheximide antibiotic was added. Fluorescence intensities of the cultures were monitored and protein concentration was estimated at 30 minute intervals. Change in fluorescence intensity per mg protein was calculated and the results were expressed as %. The experiment was repeated in three independent sets.

### 3.2.3 Construction of chimeric plasmids

Yeast expression vector pUG35 and pUG46 expressing yEGFP gene under *ADH* promoter was used for engineering degron-target protein fusions (Fig. 3.2). The oligonucleotide primers used in this study were mentioned in table 3.2.

**Table 3.2 List of oligonucleotide primers used in this study. Bold letter indicate restrictions sites**

| Gene | Oligonucleotide sequences | Size of gene |
|---|---|---|
| N50 FR | 5'-AATAAT**GGATCC**ATGTCTAGTACTCAAGTAGGAAATGCT-3' | 150 bp |
| N50 RE | 5'-AGTTGC**GAATTC**TTCCAAATCTTGGTTATTCTTTAGTTC-3' | |
| α/β FR | 5'-ATATTA**GGATCC**ATGCACAATCTTTTGCTTGAACTAAAG-3' | 924 bp |
| Nα/β RE | 5'-AAGATA**GAATTC**CATTGCTTCATTCTCAGACAGTTTTC-3' | 1038 bp |
| mODC FR | 5'-GAA**AAGCTT**GC TTC CCG CCG GAG GTG-3' | 114 bp |
| mODC RE | 5'-GGG**CTCGAG**CTACACATTGATCCTAGCAGA-3' | |
| *URA3* FR | 5'- AATGCC**GAATTC**ATGTCGAAAGCTACATATAAGG-3' | 801 bp |
| *URA3* RE | 5'- ATTATA**GAATTC**GTTTTGCTGGCCGCATCTTC-3' | |

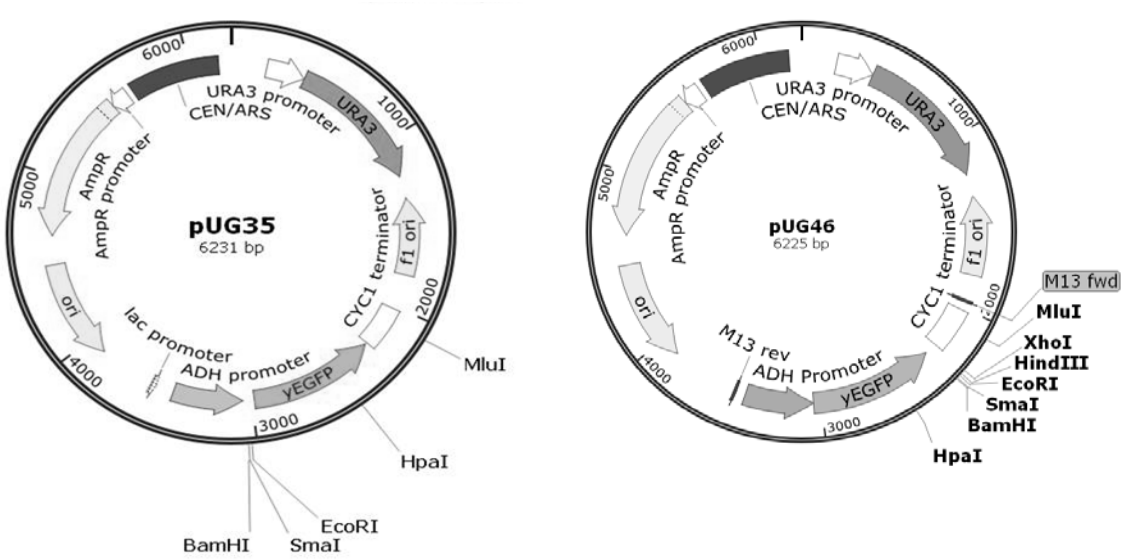


**Figure 3.2 The vector map of pUG35 and pUG46 plasmids**

### 3.2.3.1 Strategy to tag yODC degrons at N-terminal of target protein

DNA sequences for the degrons N50, α/β and Nα/β were cloned in pUG35 plasmid upstream to *yEGFP* gene in between *Bam*HI and *Eco*RI restriction sites to make degrons-yEGFP chimeric construct. *URA3* gene was introduced into the *Eco*RI site of pUG35 vector. The sequences for degrons were inserted before the fusion gene for Ura3-yEGFP, making a chimeric construct of degron-Ura3- yEGFP.

### 3.2.3.2 Strategy to tag yODC and mODC degrons at N & C-terminal of target protein

Initially DNA sequence for the degron CmODC was cloned in pUG46 vector downstream to *yEGFP* gene by using *Hind*III and *Xho*I restriction enzymes, making a pUG-yEGFP-CmODC chimeric construct. From this chimeric construct yEGFP-mODC gene was isolated from *Hpa*I and *Mlu*I sites and cloned into pUG35-Nα/β plasmid. The

resultant plasmid carries a chimeric fusion encoding yEGFP tagged with Nα/β at N-terminal and mODC at C-terminal.

Restriction digestions and ligations were done using standard methods (Sambrook, 1989). All the constructs were finally confirmed by DNA sequencing.

### 3.2.4 Fluorescence spectroscopy

Fluorescence intensities of the cultures expressing yEGFP and its chimeric fusions with degrons were monitored using Hitachi FL-7000 fluorimeter. The cells were excited at 488 nm and fluorescence emission spectra were recorded in the range of 505 to 550 nm. *S. cerevisiae* cultures with no yEGFP were used to normalize autofluorescence of the cells. The experiment was repeated three times in independent sets and the mean values are presented with error bars. SEM values are given in the graph.

### 3.2.5 Fluorescence microscopy

Evos FLc fluorescence microscope (Invitrogen) equipped with a green filter was used to observe cells expressing yEGFP and its fusions with degrons.

### 3.2.6 Flow cytometry

Fluorescence-activated cell sorting (BD-ariaII) was used to quantify yEGFP expression. The instrument settings were as follows: The yeast single-cell population was gated on forward scattering FSC and side scattering SSC. $1.5 \times 10^4$ cells were loaded in flow cytometer and out of them 30,000 events (cells) were used to analyze yEGFP expression with a standard FITC channel (excitation 488 nm, emission 515

nm). The raw data was analyzed with FlowJo software by making a histogram plot of the FITC channel.

### 3.2.7 Western blot analysis

Yeast cells containing only reporter proteins and their fusions with degron were lysed by 3 rounds of 30 sec sonication (46% amplitude, 2sec pulse on off). Debris was removed by centrifugation. Estimate the total protein concentration by Folin-Lowry method. 50ug of total protein was loaded in 12 % (w/v) SDS-PAGE (Laemmli, 1970), proteins were transferred to nitro cellulose membranes. The membrane was then blocked by blocking buffer containing 5% (w/v) nonfat dry milk in PBS-T for 1 h and than membrane was probed by primary antibody against respective proteins. Incubate the membrane overnight in the primary antibody at 4℃. Wash the blot with PBS-T four times. The membrane was incubated in HRP-conjugated secondary antibody for 1hr at room temperature. After incubation washes were given to the blot with PBS-T six times and with PBS twice. Finally blot was developed using ECL reagents (Milipore).

Western blot analysis of yeast cells containing only yEGFP and degron-yEGFP chimeric proteins were performed by using rabbit anti-yEGFP monoclonal antibody (1:5000 dilutions, Molecular Probes). Anti-yEGFP antibody was used in the western blot of Ura3-GFP control and degron-Ura3-GFP fusions. Rabbit anti-β galactosidase antibody (fluorescein isothiocyanate; Novus Biologicals) was used (1:10000 dilution), in β galactosidase experiment. Purified mouse anti-actin antibody (BD transduction laboratories) was used at 1:1000 dilution for internal control. Anti-rabbit and anti mouse horse radish peroxidise (HRP) conjugated antibody (1:5000 dilution) was used as a secondary antibody.

**Table 3.3 Proteins and their molecular weight**

| **Proteins** | **Molecular weight of protein (kD)** |
|---|---|
| yEGFP | 28.8 |
| Ura3 | 29.3 |
| β-galactosidase | 90 |
| N50-yEGFP | 33.4 |
| α/β-yEGFP | 62.4 |
| Nα/β- yEGFP | 66.5 |
| Ura3-yEGFP | 56 |
| N50-Ura3-yEGFP | 62.6 |
| Nα/β- Ura3-yEGFP | 95.7 |
| N50-β-galactosidase | 95.5 |
| Nα/β- β-galactosidase | 129.7 |
| yEGFP-CmODC | 30.8 |
| Nα/β- yEGFP-CmODC | 70.5 |

## 3.3 Results

### 3.3.1 Tagging of yODC degrons to N-terminal of target proteins

#### 3.3.1.1 Incorporation of DNA sequences representing degrons of yODC upstream to sequences of target proteins

Gene fragments corresponding to yODC degrons N50, α/β and Nα/β were successfully amplified from pRS314 containing yODC gene. After successful amplification of PCR amplicons (Fig. 3.3A), they were cloned into pUG35 yeast expression vector upstream to *yEGFP* gene. Positive constructs were confirmed by double digestion of the plasmids with *Bam*HI and *Eco*RI restriction enzymes (Fig 3.3B).

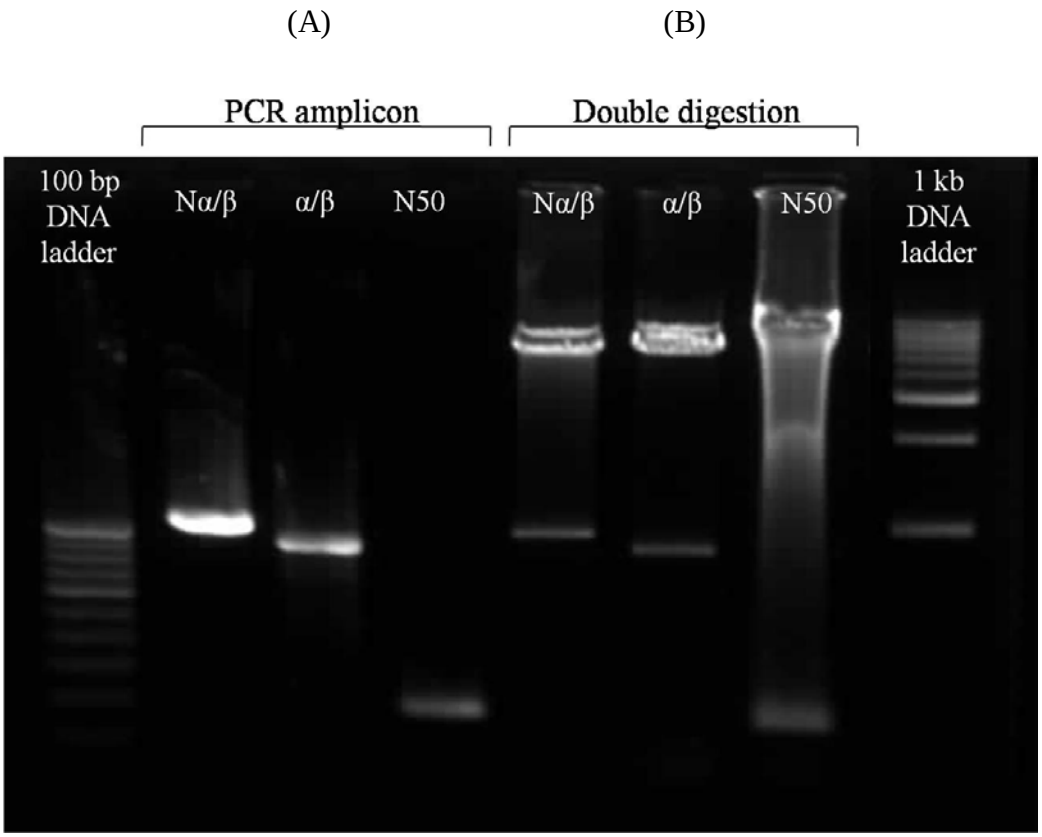


**Figure 3.3 Confirmation of chimeric constructs Nα/β-yEGFP, α/β-yEGFP and N50-yEGFP.** (A) PCR amplicons of Nα/β, α/β and N50 degrons by using gene specific primers.(B) Insert release of Nα/β, α/β and N50 degrons after double digestion with *Bam*H1 and *Eco*RI restriction enzymes.

*URA3* gene was introduced into the *Eco*RI restriction site of pUG35 vector upstream to *yEGFP* gene. The sequences for degrons N50 and Nα/β were inserted before the fusion gene for Ura3-yEGFP, making a chimeric construct of degron-Ura3- yEGFP. Fig. 3.4 shows insert release of *URA3* gene after digestion with *Eco*RI restriction enzyme.

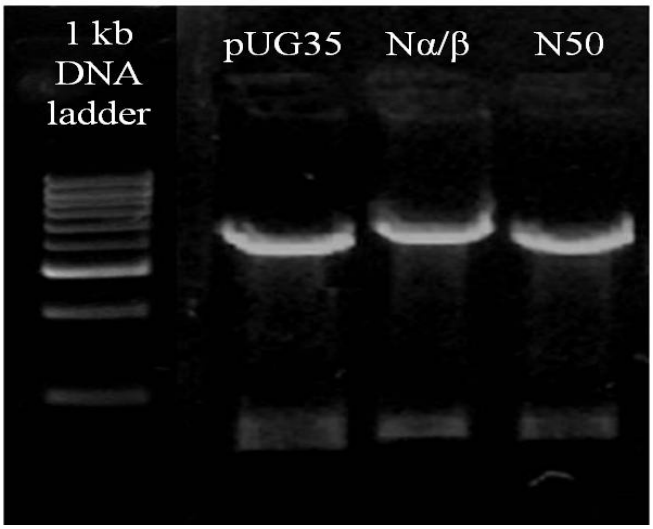


**Figure 3.4 Confirmation of chimeric constructs Ura3-yEGFP, Nα/β-Ura3-yEGFP, and N50-Ura3-yEGFP.**

All the constructs were finally confirmed by DNA sequencing. After getting positive chimeric constructs, they were transformed in *S. cerevisiae* strains Y05034 and Y651.

### 3.3.1.2 Expression of degrons as chimeric proteins of yEGFP and monitoring steady state levels of the chimeric fusions

To investigate potential to act as degrons, the chimeric fusions N50-yEGFP, α/β-yEGFP and Nα/β-yEGFP are expressed in Y05034 strain of *S. cerevisiae* (Fig. 3.5A). The fluorescence intensities of yEGFP tagged proteins in the cultures, grown to mid-log phase, were monitored initially to compare their degradation potentials. Fluorescence

spectra were recorded by exciting cells at 488 nm. Emission of yEGFP control and the peptide-yEGFP fusions was recorded at 517 nm. Fluorescence intensities of cultures expressing N50, α/β and Nα/β peptide sequences in chimeric fusion with yEGFP are 19.8%, 38.5% and 10.3% respectively when compared to control culture expressing yEGFP (Fig. 3.5B). The degron Nα/β showed highest efficiency in reducing the steady state levels of yEGFP fusion, followed by N50 and α/β. Further, the cultures were examined using flow cytometry and the result was confirmed (Fig. 3.5C).

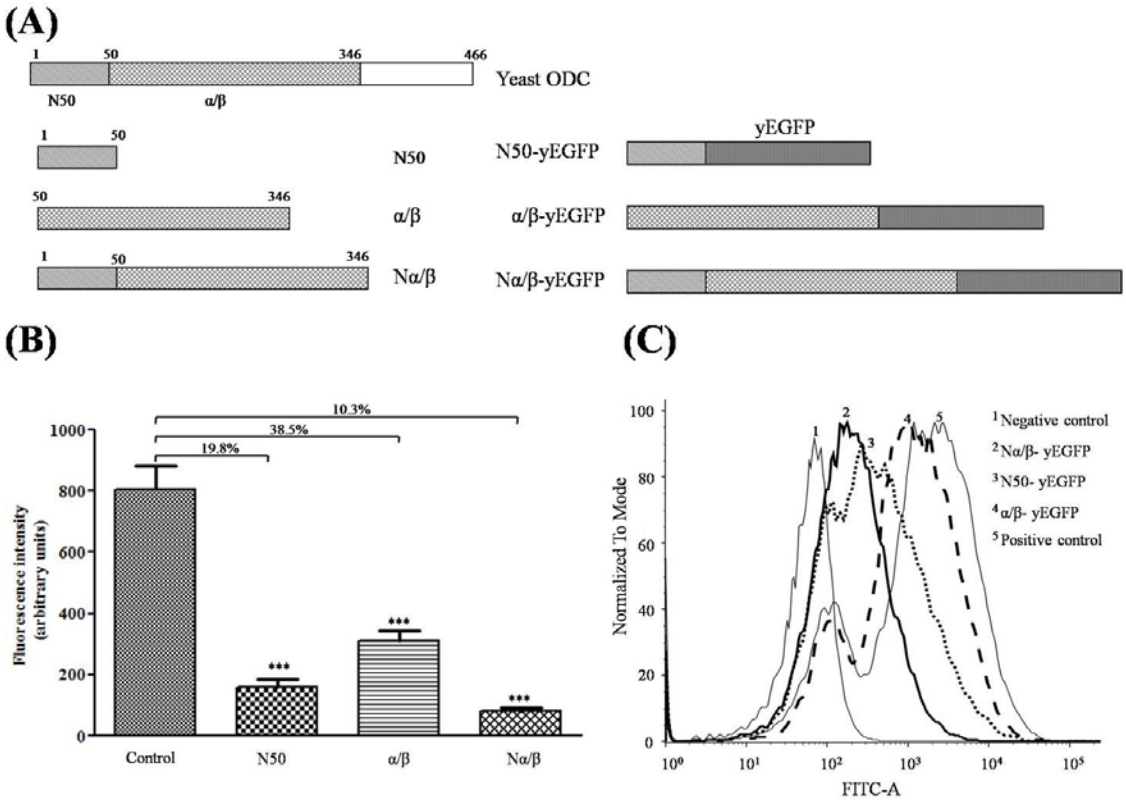


**Figure 3.5 Monitoring of steady state level of yEGFP and degrons-yEGFP fusion.**

(A) Pictorial representation of yODC, peptides N50, α/β and Nα/β (representing degrons of yODC) and their chimeric proteins with yEGFP.

(B) Comparison of $\lambda_{max}$ of fluorescence emission of yEGFP control and the peptide-yEGFP fusions (n=3 and ***p<0.001).

(C) Flow cytometric analysis of cells expressing the peptide-yEGFP chimeric fusions and control yEGFP.

#### 3.3.1.3 The effect of cycloheximide on the degradation of yEGFP and chimeric yEGFP tagged with N50, α/β and Nα/β

Cycloheximide is an inhibitor of translation in yeast and other eukaryotes. To answer the question of whether the differences seen in the steady state levels of the chimeric fusions of N50, α/β and Nα/β with yEGFP are due to the differences in their rates of degradation or due to differences in their rates of synthesis, the cultures of Y651 and Y05034 strain of *S. cerevisiae* expressing the chimeric proteins were incubated with and without cycloheximide (Fig. 3.6 A & B). Fluorescence intensity of the cultures was monitored and protein concentration was estimated at 30 minute intervals. Change in fluorescence intensity per mg protein was calculated and the results were expressed as %. The experiment was repeated in three independent sets. In the absence of cycloheximide fluorescence intensity of the culture expressing control yEGFP showed increase with time, while cultures expressing α/β-yEGFP decreased marginally. N50-yEGFP and Nα/β-yEGFP showed considerable decrease in fluorescence intensity with time. The effect seen in the absence of cycloheximide treatment with N50-yEGFP and Nα/β-yEGFP, is more pronounced with greater decrease in fluorescence intensity. In the background of inhibition of protein synthesis, the decrease in fluorescence intensity with Nα/β-yEGFP was more than with N50-yEGFP indicating the greater degradation potential of the degron Nα/β over N50. In order to have better clarity over the degradation potentials of the constructs we compared the half-lives of control yEGFP, N50-yEGFP, α/β-yEGFP and Nα/β-yEGFP, under conditions of cycloheximide treatment and antizyme induction in the above experiment. The half-lives of N50-yEGFP and Nα/β-yEGFP were 86.6 min and 58.9 min respectively. The results were extrapolated to get an approximate estimate of the half-lives of α/β-yEGFP and yEGFP, which were around 3 h and 4 h respectively. Further, the plateau seen with Nα/β-yEGFP curve between 90 min and 120 min points in

the direction of involvement of another protein namely antizyme, the synthesis of which was also negatively influenced by the inhibitor. Cycloheximide treatment on the other hand produced slight decrease in fluorescence, almost to an equal degree in cells expressing control yEGFP and α/β-yEGFP (Fig. 3.6 B & C).

To confirm the effect of antizyme induced by putrescine on the degradation rates of degrons, the two strains of yeast Y05034 and Y651 expressing yEGFP and degron-yEGFP fusions were monitored using fluorescence spectroscopy. The cultures of Y05034 were grown in presence of 1 mM putrescine. The experiment was carried out similar to the cycloheximide experiment and change in fluorescence intensity per mg protein was calculated and the results were expressed as % (Fig. 3.6A).

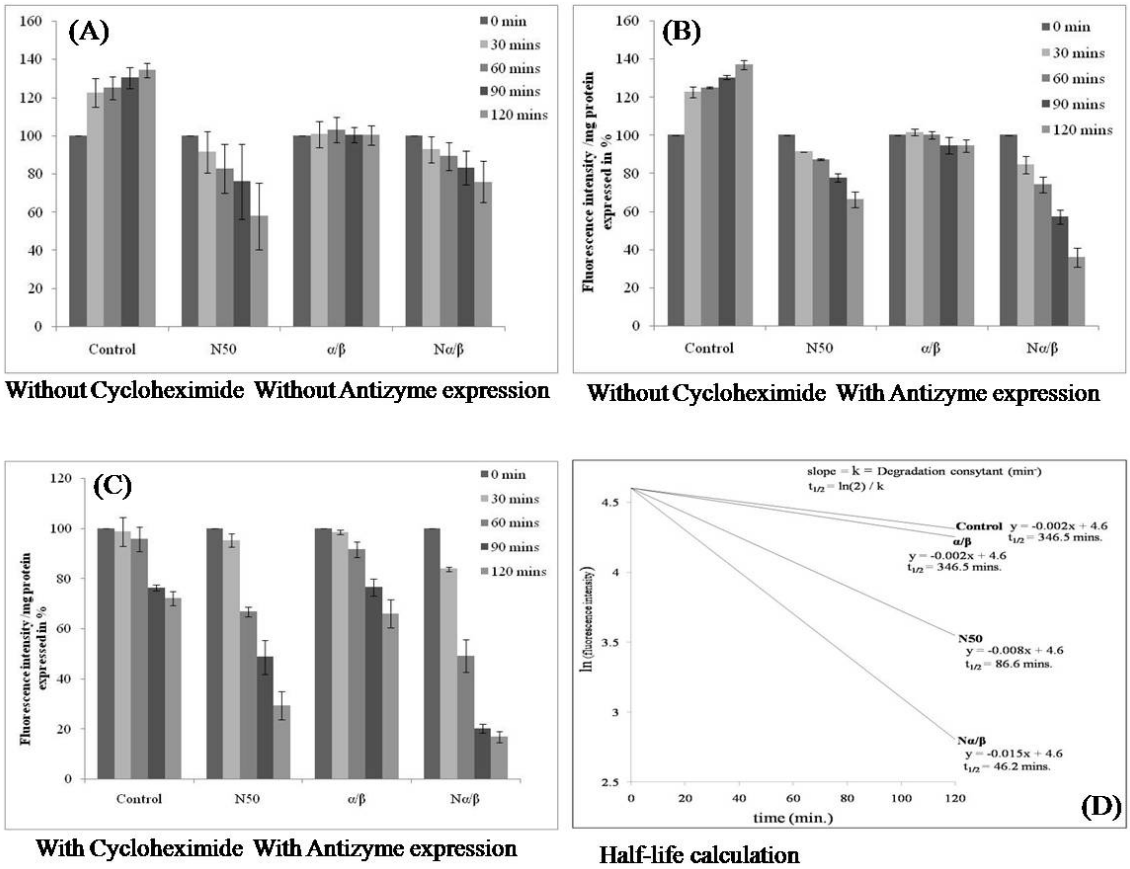


**Figure 3.6 Degradation of yEGFP tagged with N50, α/β and Nα/β with time, monitored in the presence and absence of (i) antizyme and (ii) cycloheximide.**

### 3.3.1.4 The effect of proteasomal inhibitor MG132 on the degradation of target proteins and target proteins tagged with N50, α/β and Nα/β

Yeast ODC is rapidly degraded by proteasomes. In this study, the degrons of ODC were tagged to yEGFP, Ura3 and β-galactosidase at N-terminal. To confirm the proteasomal degradation of degrons tagged target proteins yEGFP, Ura3 and β-galactosidase, the cultures of Y05034 expressing the chimeric proteins were treated with the proteasomal inhibitor MG132.

#### 3.3.1.4.1 Studies with target protein yEGFP

Flourescence intensity measurements of the cultures after four hours of exposure to inhibitor showed accumulation of N50-yEGFP and Nα/β-yEGFP, while the levels of α/β-yEGFP and yEGFP control remained unchanged (Fig. 3.7A).

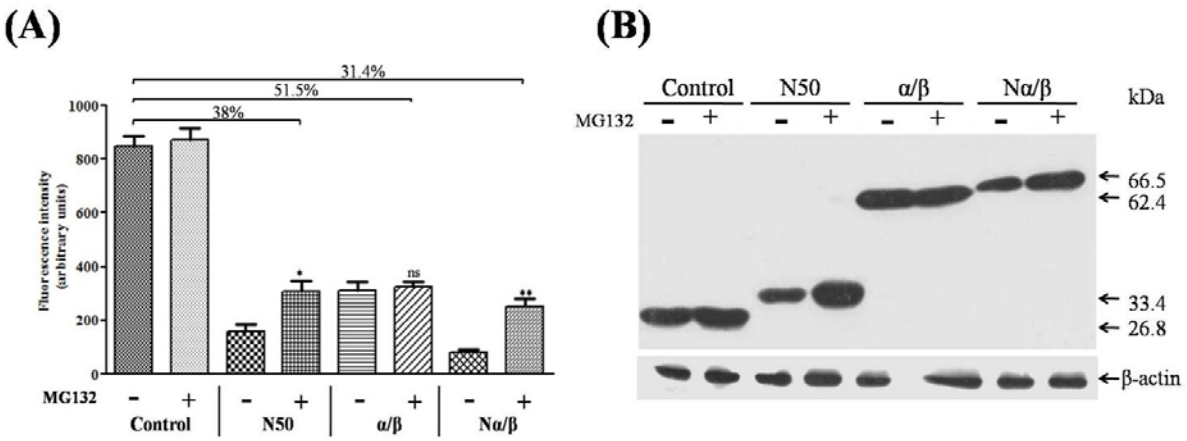


**Figure 3.7 Effect of MG132 on the degradation of chimeric protein tagged with N50, α/β and Nα/β.**

(A) Fluorescence spectroscopic analysis (n=3 and *p<0.01; **p<0.005, ns-not significant) (B) Western blot analysis of the same samples used for fluorescence studies.

Western blot analysis of the same cultures, once again showed increase in intensity of Nα/β-yEGFP and N50-yEGFP bands in the lanes containing MG132 treated cultures with respect to untreated samples, where as MG132 did not show any effect on the intensity of bands of α/β-yEGFP and yEGFP control (Fig. 3.7B). The results

indicate that both Nα/β-yEGFP and N50-yEGFP are degraded by proteasomes. However, α/β-yEGFP may not be subject to degradation by proteasomes.

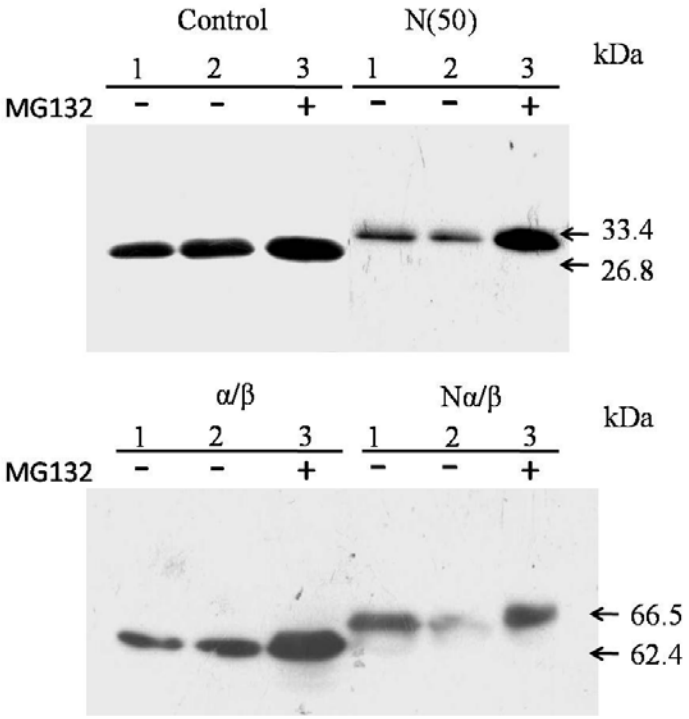


**Figure 3.8: The rate of degradation of yEGFP after being tagged with N50, α/β and Nα/β with time.**

Cells were grown in presence of 1 mM putrescine up to 0.3 $OD_{600}$ (penal 1), at this stage half of the cells were grown in same condition (penal 2) and remaining cells were treated with MG132 (penal 3). Both the cultures were collected after 4hrs.

The rate of degradation of yEGFP and degron-yEGFP fusions was compared in presence and absence of MG132 (Fig. 3.8). In absence of MG132, Nα/β-yEGFP showed maximum degradation than N50-yEGFP. While the level of yEGFP and α/β-yEGFP remained same. In presence of MG132, there was an accumulation of protein in all cases. The above results were confirmed using fluorescence microscopy (Fig 3.9).

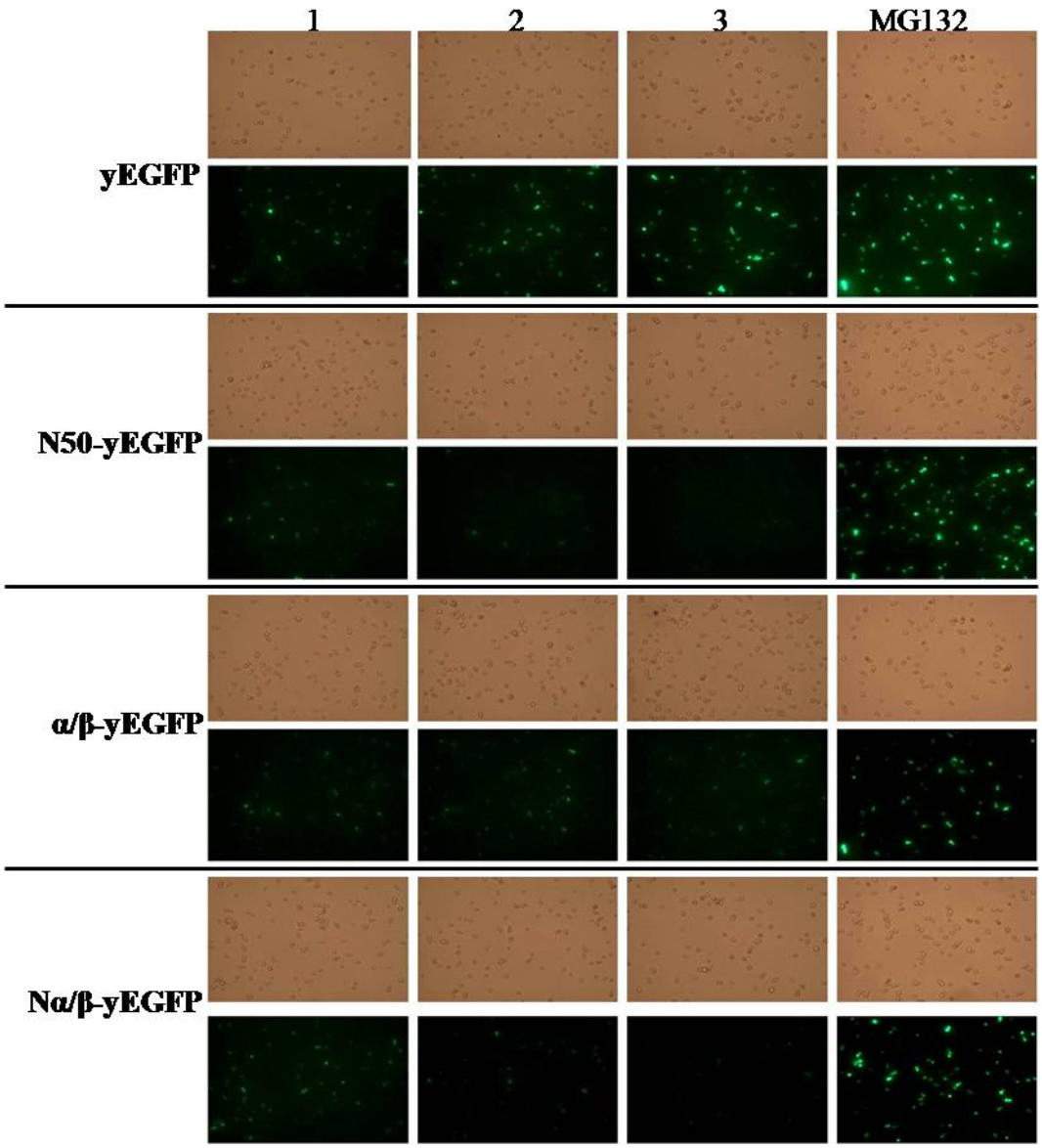


**Figure 3.9 Fluorescence microscopic studies of degrons-yEGFP chimeric constructs.** Panel 1, 2 & 3 show images of *S. cerevisiae* (BY4741) cells expressing yEGFP and degron-yEGFP chimeric fusions at different time interval.

#### 3.3.1.4.2 Studies with target protein β-galactosidase

The effect of MG132 on the degradation protein β-galactosidase was tested when it has fused to the degrons N50 and Nα/β. Constructs made in the lab earlier, N50- β-gal and Nα/β-β-gal were used in this study (Swapnali Kulkarni thesis 2012). In these constructs N50 and Nα/β were tagged to β-gal by replacing ubiquitin in pUG23 plasmid.

pUG23 plasmid (Ub-Met- β-gal) was used as a negative control. The enzyme β-galactosidase was induced using 2% galactose.

Control β-galactosidase remained unaffected both in presence and absence of MG132, whereas degradation of β-galactosidase fusions of N50 and Nα/β were inhibited (Fig. 3.10). In absence of MG132, protein level of Nα/β-β-gal was more decreased compared to N50- β-gal. The results indicate that both chimeric fusions are degraded by proteasomes. Moreover, degrons Nα/β showed better degradation than N50.

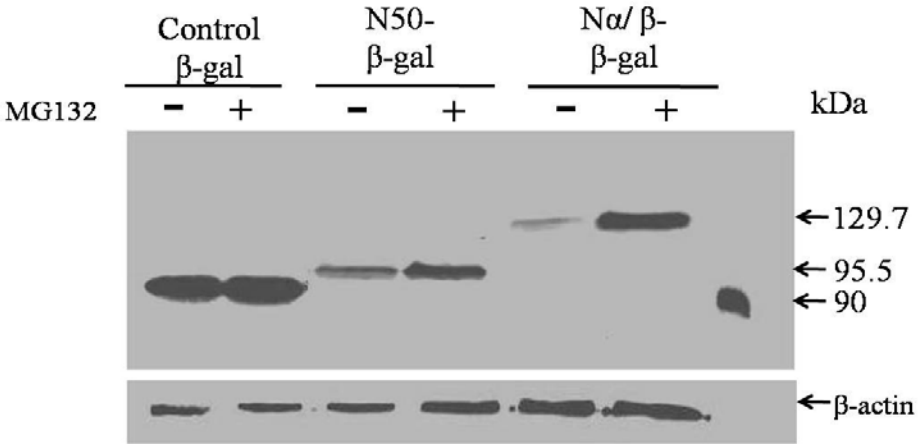


**Figure 3.10 Western blot analysis of degradation of β-galactosidase, N50- β-galactosidase and Nα/β- β-galactosidase in presence and absence of MG132.**

#### 3.3.1.4.3 Studies with target protein Ura3

Generally, Ura3 (Orotidine 5'-monophosphate decarboxylase) is used as selection marker in yeast. In this study the degradation of Ura3 was tested after it was tagged with degrons of yODC. Initially Ura3 was fused to yEGFP at N-terminus to make Ura3-GFP fusion protein. N50 and Nα/β were tagged to Ura3-GFP at N-terminal and expressed as a N50-Ura3-GFP and Nα/β-Ura3-GFP. The degradation of Ura3 was monitored in presence and absence of MG132 and Antizyme (OAz1).

As in the previous case with yEGFP, control Ura3-GFP remained unaffected both in presence and absence of MG132, whereas degradation of N50-Ura3-GFP and Nα/β-Ura3-GFP were inhibited (Fig. 3.11, MG132 penal). Chimeric fusion Nα/β-Ura3-GFP shows better degradation than N50-Ura3-GFP.

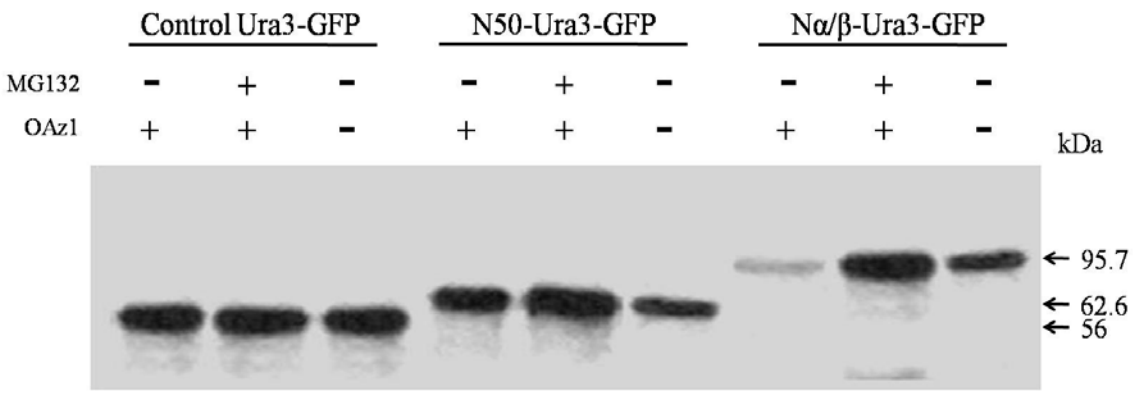


**Figure 3.11 Effect of MG132 and antizyme (OAz1) on the degradation of chimeric proteins Ura3-GFP, N50-Ura3-GFP and Nα/β-Ura3-GFP.**

### 3.3.1.5 Effect of antizyme on the degradation of proteins yEGFP and Ura3 tagged at the N-terminal with the peptides N50, α/β and Nα/β

Increased intracellular pools of polyamines lead to synthesis of the protein antizyme. Antizyme binds monomers of ODC and presents them to proteasomes for degradation, in feedback mechanism of regulation that brings down ODC levels. The two chimeric proteins Nα/β-yEGFP and α/β-yEGFP contain antizyme binding element (AzBE) in their sequence. The question was whether AzBE in the peptides Nα/β-yEGFP and α/β-yEGFP would successfully be recognized by antizyme for binding. Initially to answer the question, the cultures of Y05034 expressing yEGFP and chimeric yEGFP proteins were grown with increasing concentrations of putrescine and monitored the fluorescence intensity of cultures in mid-log phase (Fig. 3.12A). Increased polyamine levels caused considerable decrease only in the case of Nα/β-yEGFP,

establishing that the sequence in the peptide adopts a conformation that can readily be recognized and bound by antizyme for degradation. However, the slight decrease that was observed in the case of α/β-yEGFP cannot be overlooked. As expected, the yEGFP and N50-yEGFP levels remained unaffected. Putrescine concentrations used in this study are in the range of 1μM to 1mM. The strains Y651 and Y05034, which are derivatives of BY4741 can tolerate 1 mM putrescine without suffering any toxic effects of the polyamine.

In order to have a second line of evidence on the contribution of antizyme to targeted degradation, compared the levels of chimeric proteins in the antizyme deletion strain Y651 to those in Y05034 strain. The results of fluorescence intensity measurements of the cultures (Fig. 3.12B) and western blots of the cultures (Fig. 3.12C) demonstrated that Nα/β-yEGFP with two degradation signals working in tandem has better degradation potential over the other two degrons. Further, its degradation potential can easily be regulated by varying polyamine levels. A weak degron α/β, was rendered slightly more effective by high levels of antizyme. The experiment was repeated with N50-Ura3-yEGFP and Nα/β-Ura3-yEGFP fusion. Ura3-yEGFP fusion was negative control. Nα/β-Ura3-GFP fusion was degraded to a greater degree than N50-Ura3-yEGFP in Y05034 strain, when antizyme was expressed under the influence of putrescine (Fig 3.11).

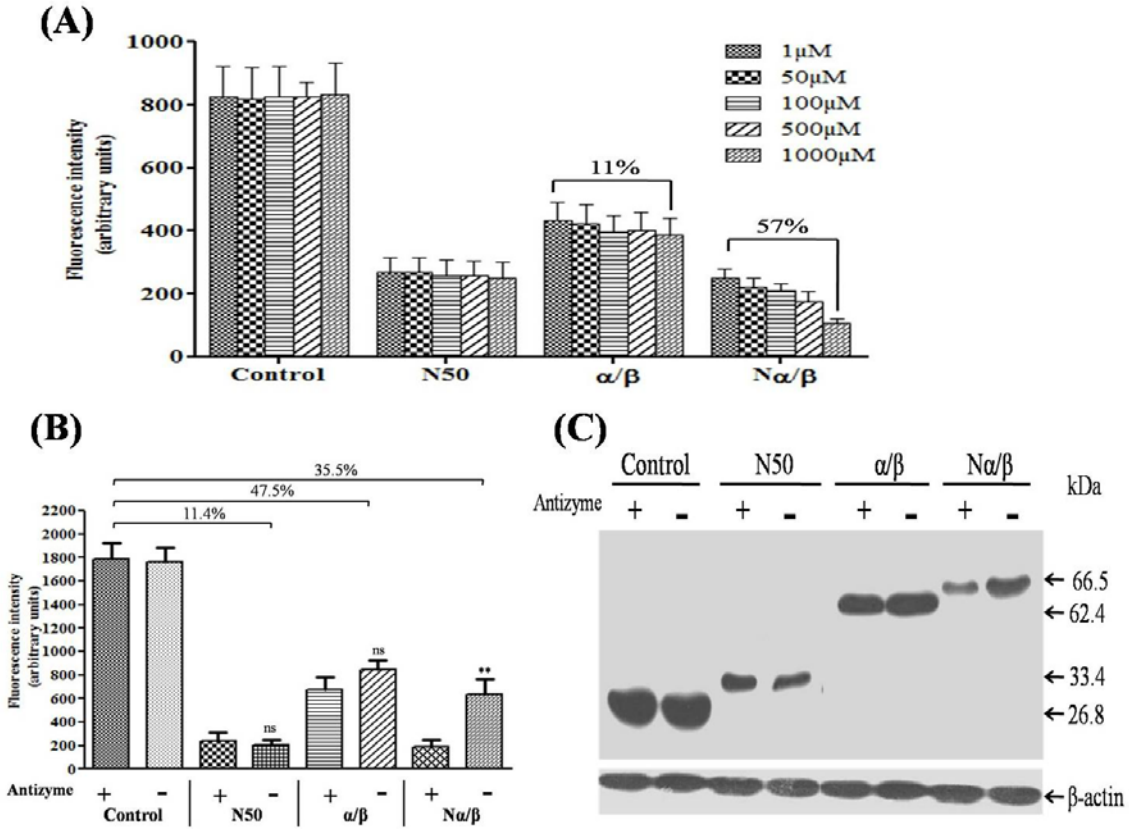


**Figure 3.12 Effect of antizyme on the degradation of proteins tagged with the peptides N50, α/β and Nα/β.**

(A) Fluorescence intensity measurements of Y05034 cultures expressing yEGFP and degrons-yEGFP fusion protein in different concentrations of putrescine (1 μM to 1000 μM), n=3

(B & C) Fluorescence intensity measurements and western blot analysis of yEGFP and degron-yEGFP fusion proteins in the presence (+) and absence (−) of antizyme respectively, n=3 and **p<0.005, ns-not significant.

### 3.3.2 Tagging of yODC and mODC degrons to C-terminal and N & C- terminals of target protein (yEGFP)

#### 3.3.2.1 Incorporation of DNA sequence representing degrons of ODCs at upstream and downstream to target protein

DNA fragment of gene coding for last 37 amino acids of mouse ODC (CmODC) was successfully cloned downstream to *yEGFP* gene in pUG46 yeast expression vector. The cloning strategy was described in section 3.2.3.2, yODC degron Nα/β and mODC degron CmODC were successfully incorporated upstream and downstream to *yEGFP* gene respectively (Fig. 3.13).

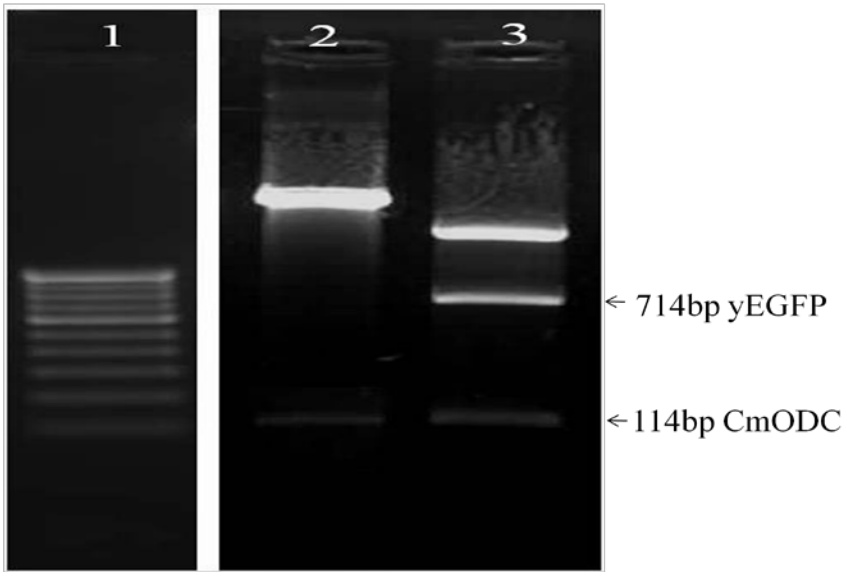


**Figure 3.13 Confirmation of chimeric constructs yEGFP-CmODC and Nα/β-yEGFP-CmODC.**

Lane 1 shows 1kb DNA ladder, Lane 2 shows insert release of CmODC after digestion with *Hind*III and *Xho*I restriction enzymes, Lane 3 shows insert release of yEGFP and CmODC after digestion with *Eco*RI and *Xho*I restriction enzymes.

### 3.3.2.2 Monitoring steady state levels of yEGFP, yEGFP-CmODC and Nα/β-yEGFP-CmODC fusions

To investigate potential to act as degrons, the chimeric fusions yEGFP-CmODC and Nα/β-yEGFP-CmODC are expressed in Y05034 strain of *S. cerevisiae* (Fig. 3.14A). It has reported earlier that attachment of only CmODC to other stable proteins caused their degradation via proteasome (Zhang et al., 2003). In this study CmODC was taken as positive control.The fluorescence intensities of yEGFP tagged proteins in the cultures, grown to mid-log phase, were monitored initially to compare their degradation potentials (Fig. 3.14B). The results indicate that degron Nα/β-CmODC showed highest efficiency in reducing the steady state levels of yEGFP (90.1%) than CmODC (81%). Further, the cultures were examined using flow cytometry and the result was confirmed (Fig. 3.14C).

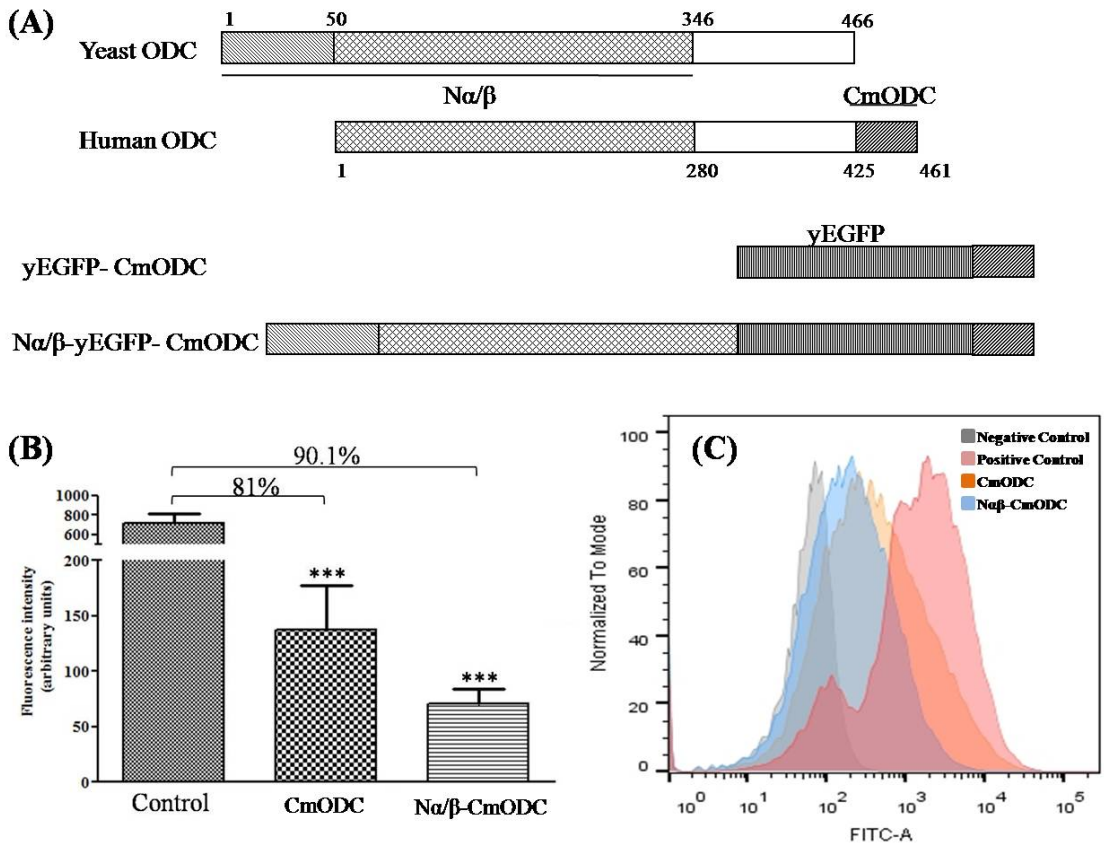


**Figure 3.14 Monitoring steady state levels of yEGFP, yEGFP-CmODC and Nα/β-yEGFP-CmODC fusions.**

(A) Pictorial representation of yeast and mouse ODC and their degron expressed as chimeric fusions with yEGFP

(B) Comparison of $\lambda_{max}$ of fluorescence emission of yEGFP control and yEGFP-CmODC and Nα/β-yEGFP-CmODC fusions (n=3 and ***p<0.001).

(C) Flow cytometric analysis of cells expressing yEGFP yEGFP-CmODC and Nα/β-yEGFP-CmODC fusions, where in yEGFP was used as a positive control and Y05034 cells without expressing yEGFP as a negative control

#### 3.3.2.3 The effect of proteasomal inhibitor MG132 on the degradation of yEGFP, yEGFP-CmODC and Nα/β-yEGFP-CmODC fusions

As mentioned earlier, ODCs of yeast and mouse are rapidly degraded by proteasomes. To test whether the chimeric fusions yEGFP-CmODC and Nα/β-yEGFP-CmODC are degraded by proteasomes, the log phase cultures of Y05034 strain of *S. cerevisiae* were incubated with the proteasomal inhibitor MG132. Flourescence intensity measurements of the cultures after four hours of exposure to inhibitor showed accumulation of yEGFP-CmODC and Nα/β-yEGFP-CmODC, while the levels of control yEGFP remained unchanged (Fig. 3.15A). Western blot analysis of the same cultures, once again showed increase in band intensity of Nα/β-yEGFP-CmODC and yEGFP-CmODC in the lanes containing MG132 treated cultures with respect to untreated samples (Fig. 3.15B). Same cultures were analyzed by flow cytometry (Fig. 3.15C). The results indicate that both Nα/β-yEGFP-CmODC and yEGFP-CmODC are degraded by proteasomes.

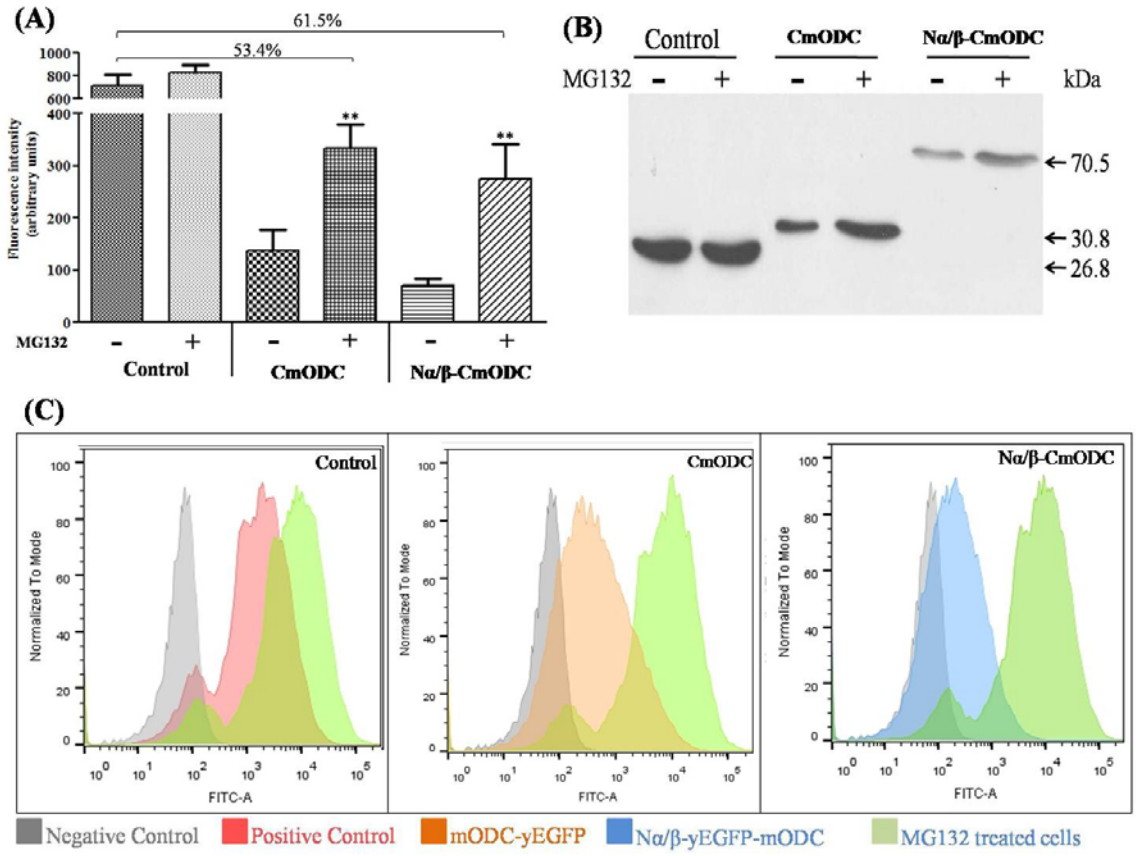


**Figure 3.15 Effect of MG132 on the degradation of yEGFP, yEGFP-CmODC and Nα/β-yEGFP-CmODC fusions**

(A) Fluorescence spectroscopic analysis of cells expressing yEGFP, yEGFP-CmODC and Nα/β-yEGFP-CmODC fusions (n=3 and ; **p<0.005) (B) Western blot analysis of the same samples used for fluorescence studies. (C) Flow cytometric analysis of same sample

### 3.3.2.4 The effect of antizyme on the degradation of yEGFP, yEGFP-CmODC and Nα/β-yEGFP-CmODC fusions

As mentioned earlier, Nα/β peptide work as an independent degron and its degradation can be regulated by antizyme protein. Same experiment was carried out to check the effect of antizyme on the combination of degrons Nα/β and CmODC and the degradation mediated by them by expressing yEGFP, yEGFP-CmODC and Nα/β-yEGFP-CmODC fusions in antizyme deletion strain Y651. Fluorescence intensity measurement and western blot analysis results indicate that degradation of Nα/β-

yEGFP-mODC fusion was affected in the absence of antizyme. While the level of yEGFP and yEGFP-cmODC fusion remained same in the presence and absence of antizyme. It has established that antizyme can interact with Nα/β-yEGFP-CmODC fusions and rapidly degrade it through proteasome.

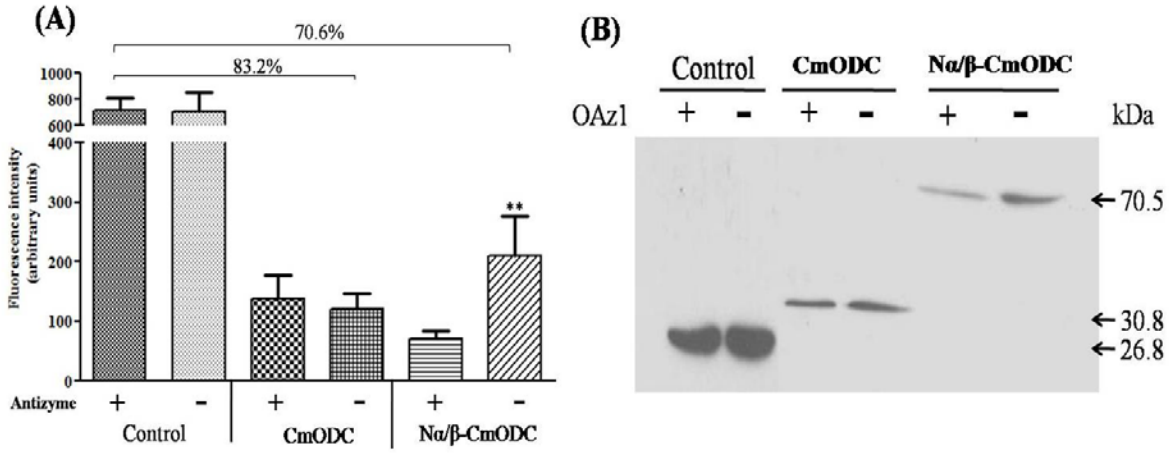


**Figure 3.16 Effect of antizyme on the degradation of yEGFP, yEGFP-CmODC and Nα/β-yEGFP-CmODC fusions**

(A) Fluorescence spectroscopic analysis of cells expressing yEGFP, yEGFP-CmODC and Nα/β-yEGFP-CmODC fusions (n=3 and **p<0.005) (B) Western blot analysis of the same samples using rabbit anti-GFP antibody.

## 3.4 Discussion

Present study demonstrated the potential of selected degrons of ODCs to act as efficient vehicle for targeted protein degradation. Initially three degrons N50, α/β and Nα/β of yeast ODC were tagged to yEGFP at N-terminus. The steady state level of yEGFP was monitored in Y05035 strains of *S. cerevisiae*. Among the three degrons, N50 and Nα/β tagged yEGFP shows rapid degradation in the cells. From proteasomal inhibition study, it was established that N50 and Nα/β mediate degradation of yEGFP via proteasome, while α/β failed to degrade target protein. The additional feature of Nα/β degron is that it has antizyme binding site. The degradation activity of degron

Nα/β was influenced by the presence of antizyme. Nα/β-yEGFP fusion protein shows maximum level of degradation in the cells with increased antizyme expression. In the absence of antizyme there was an accumulation of Nα/β-yEGFP protein. The degradation activity of degron N50 was not affected by antizyme expression.

Earlier it was reported that first 44 residues of yeast ODC (ODS) can act as a independent transplantable degron (Godderz et al., 2011). In this study, first 50 residues of yODC have been selected and our results support earlier observation. To confirm the efficiency of degrons N50 and Nα/β towards the targeted protein degradation, they were tagged to two more target proteins β-galactosidase and Ura3. β-galactosidase is bulky protein and Ura3 is functional regulatory protein in yeast. After tagging these two proteins with degrons N50 and Nα/β, same results were observed as in the case of yEGFP. The chimeric fusions degron-β-galactosidase and degron-Ura3 were rapidly degraded by proteasome. These results established that two peptides of yODC namely N50 and Nα/β can act as degrons without rest of the protein. Moreover, the degradation Nα/β was regulated by antizyme expression, this regulatory feature makes it an ideal degron for targeted protein degradation.

Another outcome of this study is that Nα/β is combination of N50 and α/β degrons. While α/β was failed to degrade target protein. Earlier structural results of N50, α/β and Nα/β were mentioned in chapter 2, N50 shown α-helical structure while Nα/β shown mix α/β like structure but with the deletion of N50 i.e. α/β, became β-sheet like structure. Correlating the structural results with the results of functional studies, it can be concluded that the first 50 amino acids of yeast ODC are important for its degradation. This N50 is helping in maintaining structure of Nα/β by which it can be recognized by antizyme. In yeast, truncation of its N-terminus of ODC leads to stabilization of whole protein (Godderz et al., 2011). When antizyme interact with ODC,

these N-terminal residues are exposed and they act as degradation signal of yeast ODC, similar to the C-terminal of mammalian ODC (Li and Coffino, 1993). α/β on the other hand failed to fold and therefore cannot interact with antizyme.

Further, target protein was tagged with two degrons from different host ODCs. yODC degron and mODC degron CmODC were tagged to yEGFP at N-terminal of and C-terminal respectively. This Nα/β-yEGFP-CmODC chimeric fusion shows better degradation over Nα/β and it can also regulated by antizyme expression.