CHAPTER 1

INTRODUCTION

Introduction

1.1 Protein degradation

Protein molecules are continuously synthesized and degraded in all living organisms. Protein degradation performs an important role in cell metabolism. Degradation is not only meant for elimination of defective proteins, but also it is important to maintain cellular protein homeostasis. Degradation of intracellular proteins is involved in regulation of many cellular processes, such as cell cycle and division, regulation of transcription factors, recycling of amino acids and cellular protein quality control. Misfolded and damaged proteins of the cell have to be degraded to avoid toxicity (Fig. 1.1) (Chen et al., 2011).

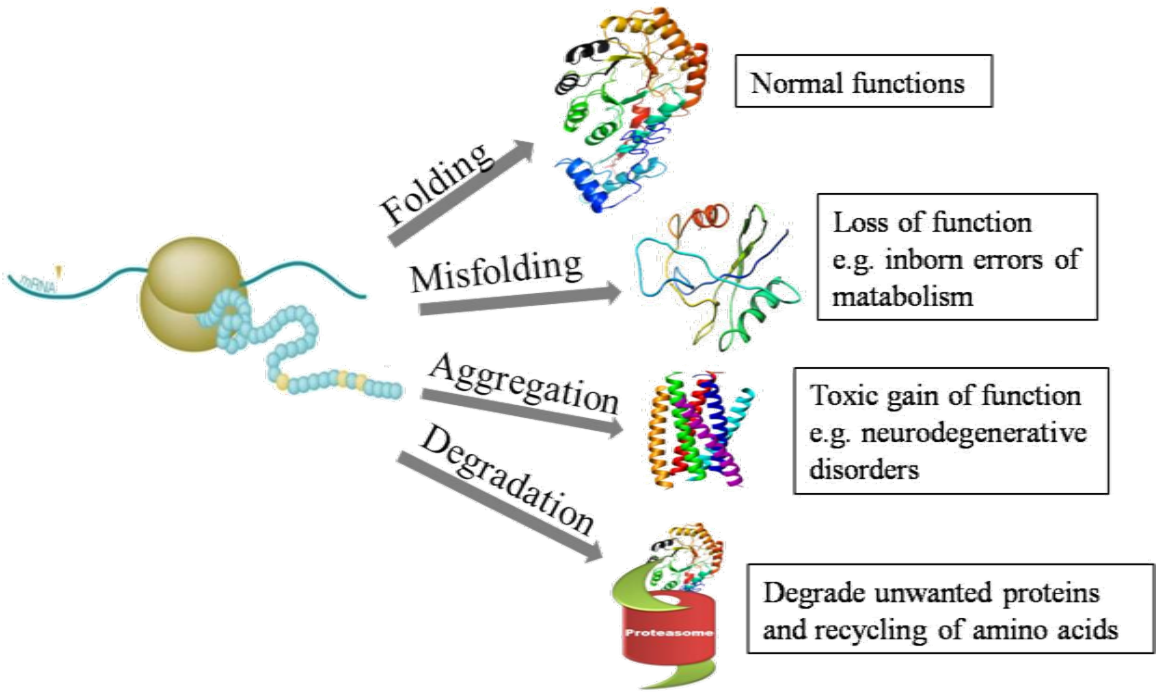

Figure 1.1 The different fates of protein after synthesis

Misfolded proteins cause inappropriate interactions with other cellular components leading to accumulation of potentially toxic protein deposits (Lansbury and Lashuel, 2006). Various diseases are characterized by the formation of such damaged or misfolded proteins. Nowadays these types of diseases are recognized as 'conformational diseases' caused by proteins taking up non-native conformations which leads to aggregation. These include diverse disorders, ranging from lysosomal storage diseases (Sawkar et al., 2006), cancer (Dai et al., 2007), cystic fibrosis (Koulov et al., 2010), many neurodegenerative disorders such as Alzheimer (AD), Parkinson's (PD), and Huntington's (HD) diseases (Caughey and Lansbury, 2003; Cohen and Kelly, 2003; Morimoto, 2008).

In the eukaryotic cells, protein degradation is mainly of two types. They are: 1) lysosomal protein degradation (De Duve and Wattiaux, 1966) and 2) proteasome mediated degradation. In the past, researchers thought that lysosomal enzymes are responsible for degrading intracellular proteins. Generally lysosome degrades those proteins which enter the cell through phagocytosis and pinocytosis. The majority of intracellular proteins in eukaryotic cells are degraded by a large multisubunit complex called the 26S Proteasome (Rock et al., 1994). In the second type of protein degradation proteasome is involved. The substrates of proteasomes are of two types, namely ubiquitinated or nonubiquitinated. Ubiquitinated substrates are tagged with the protein ubiquitin and are degraded by ubiquitin proteasome system (UPS). UPS is a pathway meant for specific protein degradation (Ciehanover et al., 1978; Etlinger and Goldberg, 1977). Majority of the cellular proteins are degraded by ubiquitin proteasome system (Chen and Dou, 2010). Ubiquitin is key regulatory molecules in UPS.

Degradation of protein is an irreversible process. Therefore, recognition of high specificity is required to avoid destruction of wrong proteins. Ubiquitin is used as a tag

for specific protein degradation. Ubiquitin is covalently attached to proteins in a polymeric form and marks them for degradation (Ciechanover et al., 1980). Along with ubiquitin there are three enzymes which catalyze ubiquitination, namely ubiquitin activating enzyme E1, ubiquitin conjugating enzyme E2, ubiquitin ligase E3 and the multi-subunit protease complex proteasome, which act in series to constitute the ubiquitin proteasome system (Bachmair et al., 1986; Weissmann, 1997). The ubiquitin proteasome system regulates removal of many intracellular proteins, including those which control cell cycle progression, apoptosis, signal transduction and induction of the inflammatory response.

Proteasome is favorable protease for selective protein degradation. Over the past decade, proteasomes have come out as one of the primary protease in the intracellular degradation of proteins. Their function has been directly or indirectly linked to a wide range of diseases ranging from cancers to neurodegenerative diseases. The proteasome is able to digest hundreds of unrelated proteins. It can also digest foreign proteins which are not synthesized in host cell. The 26S proteasome is a 2000 kDa multiprotein complex composed of a proteolytically active 20S core particle (20S proteasome) that is capped by 19S regulatory particles (19S RP). The 19S regulatory units recognize specific sequence of proteins and control access to the proteolytic core. Generally polyubiquitinated proteins are recognized by 19S regulatory particle. 19S subunit is also able to interact with other proteins besides ubiquitin. 19S regulatory particle has ATPase activity to unfold the proteins and then translocate them to the 20S core particle. The 20S core particle is composed of four stacked rings. The outer rings (upper and lower) contain seven α–subunits each and the two inner rings (middle) contain seven β–subunits each, arranged like α_{1-7} β_{1-7} β_{1-7} α_{1-7} pattern (Fig. 1.2). 20S core particle is a hollow cylindrical structure. Each β-ring is having three β-subunits with proteolytic

activity. These proteolytic active sites of 20S proteasome are prevented by the two α–rings from direct contact with the cytoplasm to avoid nonspecific protein degradation in the cell (da Fonseca and Morris, 2008). Because of the self-compartmentalized active sites and substrate specificity, proteasome is a favorite system for designing strategies for targeted protein degradation by using different recognition sequences (degrons) (Prabha et al., 2012).

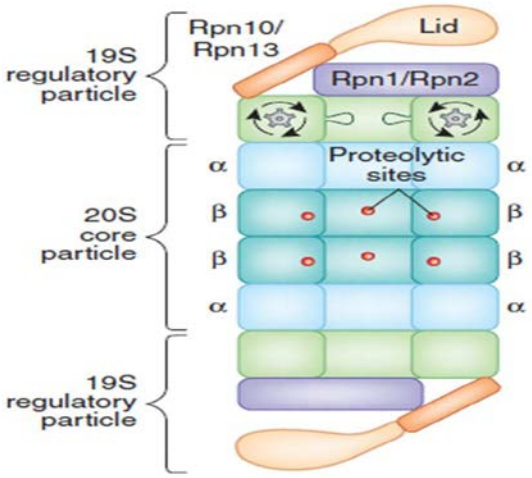

Figure 1.2: Structure of 26S proteasome

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1.2 Targeted protein degradation

In the nature protein degradation plays an important role in every basic cellular process. In proteasomal pathway some proteins are specifically targeted for degradation by degradation determining sequences which are also known as degrons. The process is called targeted protein degradation. The degrons are responsible for protein degradation by ubiquitin dependent and independent mechanisms. The selective degradation of many

short-lived proteins in the cell is mediated via the ubiquitin proteasome pathway. Ubiquitin is a 76 amino acid residue protein. It is covalently conjugated in a highly regulated multistep process to the substrate protein, marking the substrate for degradation by the 26S proteasomes (Glickman and Ciechanover, 2002; Hershko, 1996).

Here some methods for targeted protein degradation are mentioned. These methods include ubiquitin dependent and independent mechanisms.

1.2.1 Ubiquitin and Ubiquitin proteasome system used for targeted protein degradation

- Ubiquitin plays major role in specific protein degradation in the cell. Ubiquitin is conjugated through its lysine residues to the substrate proteins. Lys-29, Lys-11 and Lys-48 linked polyubiquitination is a signal for substrate protein degradation via proteasome (Jin et al., 2008; Pickart and Fushman, 2004; Thrower et al., 2000). With the help of three processing enzymes E1, E2 and E3, ubiquitin is tagged to substrate proteins.
- In eukaryotes, the N-end rule pathway is a part of the UPS. The N-end rule is regulation of in vivo half life of protein based on of its N-terminal residue. Degradation Signals (degrons) that are targeted by the N-end rule pathway called N-degrons. E3 Ub Ligases of the N-end rule pathway are called N-recognins. They bind to the primary destabilizing N-terminal residues of N-end rule substrates. Methionine is considered as most stabilizing amino acid residue while proline as most destabilizing residue (Varshavsky., 1996).
- Chimeric proteins expressed with N-terminal ubiquitin tag undergo polyubiquitination for degradation. Ubiquitin-specific processing enzymes or deubiquitinating enzymes remove the ubiquitin tag from chimeric proteins.

Removal of ubiquitin from chimeric protein depends on N-terminal destabilizing residue on ubiquitin (Prabha et al., 2012). Chimeric proteins with N-terminal proline cannot be cleaved from ubiquitin, as they are resistant to action of ubiquitin-specific processing enzymes, consequently becoming substrates for ubiquitin fusion degradation (UFD) pathway and therefore are short-lived (Bachmair et al., 1986).

- In our laboratory certain mutation of ubiquitin were constructed to study the role of conserved residues in ubiquitin mediated protein degradation. The mutant protein showed minimal changes in secondary and tertiary structures. When functional integrity of the mutant forms of ubiquitin was tested by expressing them from plasmid in *UBI4* deletion mutants of yeast (Fig.1.3A), it was observed that the half-life of the chimeric fusion of ubiquitin with β-galactosidase (Ub-Pro-β-gal) was extended selectively, where proline is the N-terminal residue of the β-galactosidase (Doshi et al., 2014).
- E2 enzymes are C terminally fused to peptide ligands of substrate proteins are able to ubiquitinate the binding partners of ligands with high specificity (Fig. 1.3B). A number of such pairs were tested for ubiquitination successfully (Gosink and Vierstra, 1995).
- Intentionally bring target protein into vicinity of E3 ligase by constructing bifunctional degrons which contain two recognition elements, one for target protein and other for E3 ligase. Target protein and E3 ligase interact with recognition elements of the degron simultaneously and target protein is polyubiquitinated and degraded via UPS. The method is known as PROTACs (PROteolysis TArgeting Chimeric molecules) (Fig. 1.3C) (Raina and Crews, 2010).

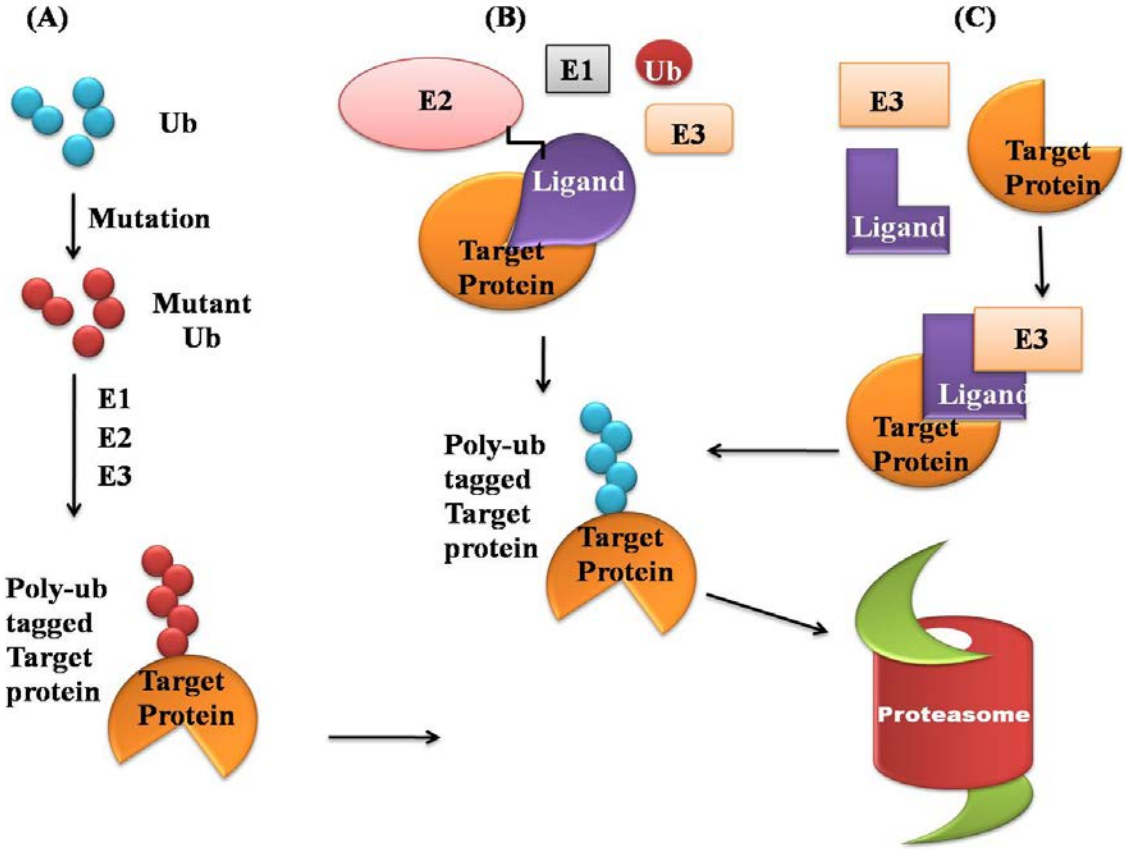

Figure 1.3 Ubiquitin dependent targeted protein degradation.

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1.2.2 Ubiquitin independent targeted protein degradation

Many proteins in the cells are degraded by ubiquitin independent mechanisms. These mechanisms can be adapted for artificially induced targeted protein degradation.

Recent discoveries in the field of protein degradation have revealed that several proteins are degraded by 26S proteasome without undergoing ubiquitination. These Ub-independent substrates include the polyamine biosynthetic enzyme ornithine decarboxylase (ODC) (Godderz et al., 2011; Li and Coffino, 1992), several

protooncoproteins and tumor suppressors (Jariel-Encontre et al., 2008), the human thymidylate synthase (hTS) (Melo et al., 2010), the gap junction protein connexin43 (Su et al., 2010), the BIMEL pro-apoptotic BH3-only protein (Wiggins et al., 2011), the transcriptional co-activator SRC-3 (Li et al., 2006), the yeast transcription factor Rpn4 (Ju and Xie, 2004) and the hepatitis C virus F and core proteins (Moriishi et al., 2003; Yuksek et al., 2009).

Ornithine decarboxylase (ODC) is one of the best examples for ubiquitin independent degradation of proteins. Some degradation signals lie within the sequence of ODC. They are recognized by another protein termed as antizyme (Az). We are working on ODC/Az system in yeast. More information regarding ODC/Az system is given in Section 1.3.

When N-terminal 44 or 50 residues of yeast ODC or C-terminal unstructured region of mouse ODC were attached to stable protein, whole chimeric fusion protein was found to be rapidly degraded by 26S proteasome (Godderz et al., 2011; Joshi et al., 2015; Zhang et al., 2003).

1.2.3 Ubiquitin dependent and independent protein degradation

SMASh (small molecule–assisted shutoff), which allows for chemically induced degradation of target proteins by controlling gene expression at the post-translational level. A chimeric construct was engineered in which the protein of interest was tagged with the hepatitis C virus protease NS3 and a destabilizing degron (**Fig. 1.4**). After translation, proteases cleave the tagged protein from the degron and NS3 itself, yielding unmodified gene product. Protease inhibitor asunaprevir was added to bring about rapid

degradation of target proteins tagged with degrons and NS3 protease (Hannah and Zhou, 2015).

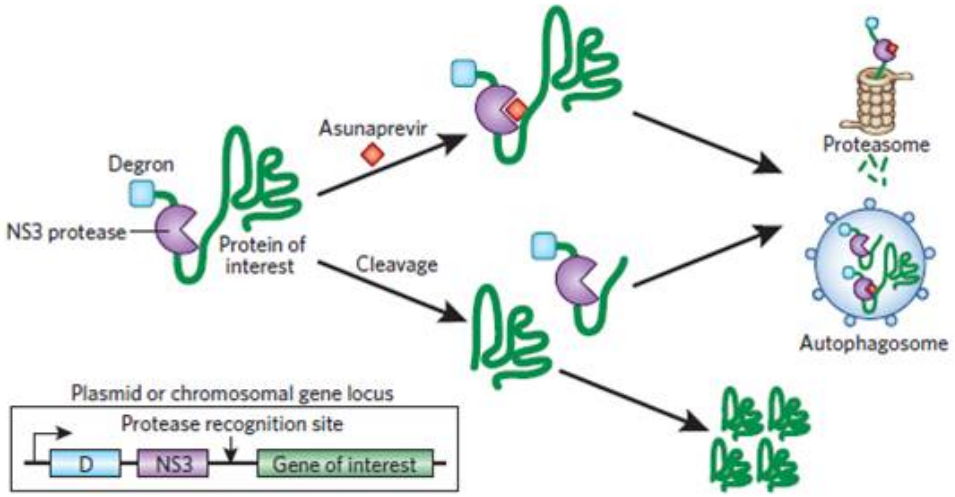

Figure 1.4: SMASh system for targeted protein degradation

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1.2.4 Advantages of targeted protein degradation

The selective degradation of intracellular proteins can be a powerful tool for protein function analysis and therapeutic applications. In the nature, targeted protein degradation occurs for replacement of regulatory proteins after they serve their function, replenishment of amino acid pools in times of starvation and generation of peptide hormones (Varshavsky, 1996). Regulatory proteins have short half-life and contain degradation signals for their degradation. Because of their degradation signals, regulatory proteins maintain their concentration in the cells resulting in down regulating the rates of synthesis of the target gene products. Researchers have developed many

methods for targeted protein degradation by modifying existing pathway. There are many advantages for targeted protein degradation: (i) It has high specificity, (ii) degrades targeted protein directly and rapidly by existing cellular pathways and (iii) expression of targeted protein can be regulated by inducer (Prabha et al., 2012).

1.3 Ornithine decarboxylase (ODC)

Ornithine decarboxylase (ODC; EC 4.1.1.17) catalyzes the first step in the polyamine biosynthetic pathway. It catalyzes the decarboxylation of ornithine to putrescine (Tabor and Tabor, 1984). Putrescine is then converted into the polyamines spermidine and spermine (Fig. 1.5). Polyamine plays important roles in both normal and neoplastic growth. Alterations in polyamine synthesis via changes in ODC level leads to tumor formation and carcinogenesis (Gerner and Meyskens, 2004; Pegg, 1988).

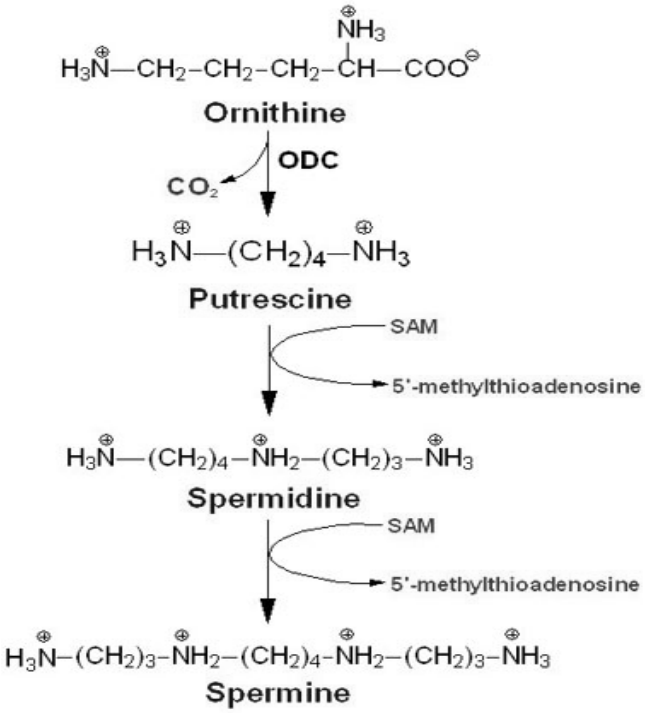

Figure 1.5 Biosynthesis pathway of polyamines

ODC is catalytically active in homodimer form, which is formed by head to tail interaction of the two monomers of ODC. It is approximately 450 amino acids long and has a molecular weight of 52 kDa. Monomeric form of ODC is not enzymatically active. ODC is one of the most rapidly degraded proteins in mammalian cells. It is forming putrescine from ornithine by decarboxylation reaction.

1.3.1 Structure

The structure of ornithine decarboxylase has two domains namely, N terminal α/β barrel domain and C-terminal β-sheet domain (Fig. 1.6) (Almrud et al., 2000; Kern et al., 1999).

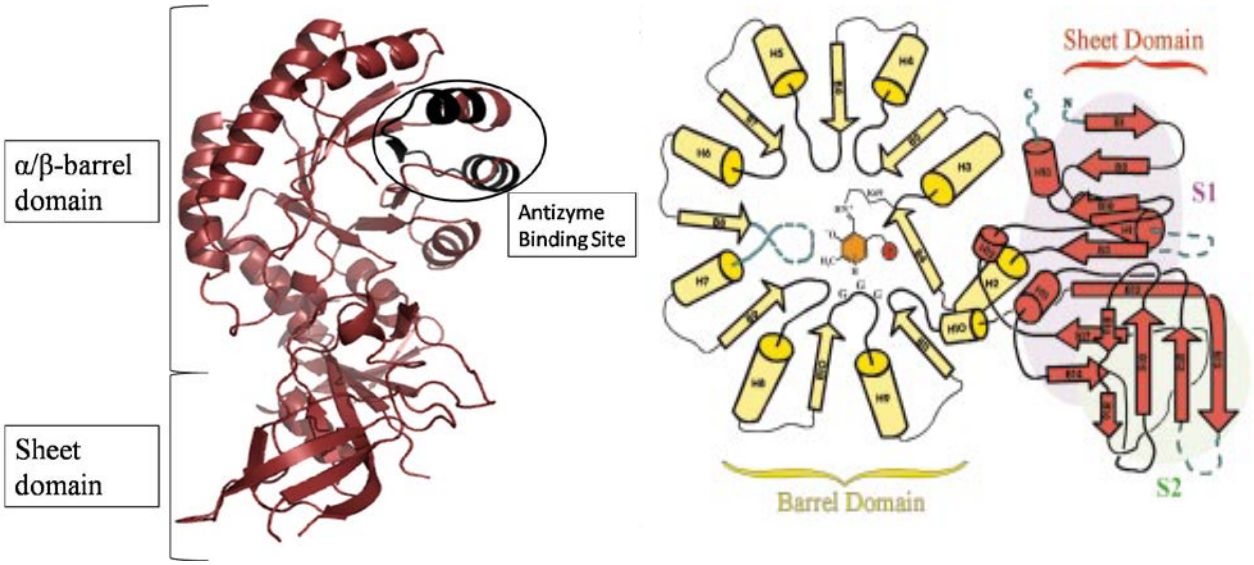

Figure 1.6 Structure of human ODC. The structure of mouse ODC resembles human ODC closely.

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According to 2.1Å crystal structure of human ODC subunit consists of two domains: a TIM-like α/β barrel (residues 46-280), and a sheet domain (residues 7-45, 281-427). α/β barrel is domain composed of 9 α Helices H2- H10, while β-sheet domain consist of two sheets S1 and S2 with two helices H11 and H12. The strands of S1 and S2 are roughly perpendicular, forming a central hydrophobic core. A primary sequence alignment and structural comparison of hODC (PDB ID: 1D7K), mODCt (PDB ID: 7ODC), and tODC (PDB ID: 1F3T) reveals that extensive sequence and structural identities are observed between ODCs of these three eukaryotic species. Yeast ODC structure is not available so far.

1.3.2 Regulation of ODC

Proteasomal degradation usually requires ubiquitin tagged substrates. But degradation of ODC is totally ubiquitin independent process (Murakami et al., 1992). Some degradation signals lie within the sequence of ODC, which are recognized by another protein termed as Antizyme (Az). Antizyme recognizes a large electropositive patch on the surface of the αβ-barrel domain of ODC monomer termed as Antizyme Binding Element (AzBE) (Kern et al., 1999) and targets it for proteasomal degradation.

Antizyme (Az) is a non-competitive inhibitor of ODC. It is synthesized by +1 ribosomal frameshifting mechanism in response to increase in polyamine concentration in the cell (Heller et al., 1976). This inhibition is due to tight binding of the antizyme with ODC monomer forming a heterodimer. This heterodimer prevents enzymatic activity of ODC. Monomer of ODC has higher affinity towards the antizyme and it prefers formation of ODC-Az heterodimer over ODC-ODC homodimer.

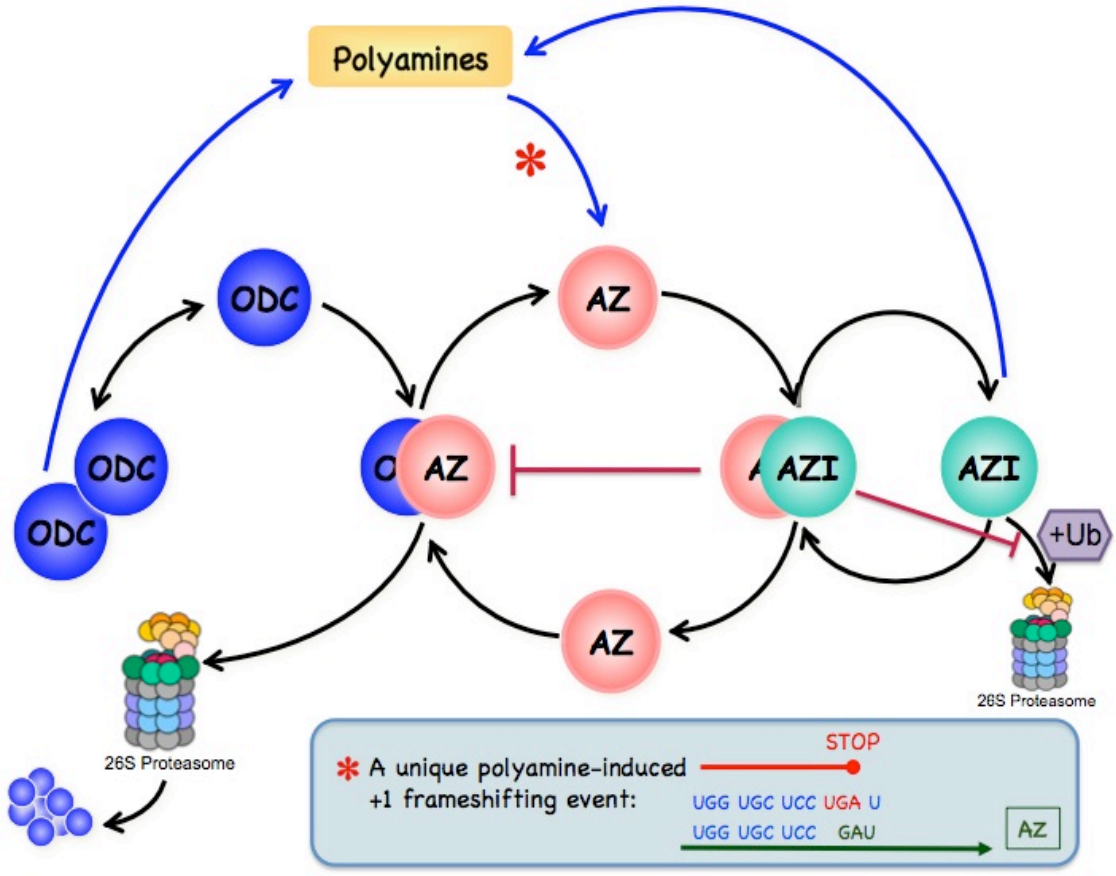


Figure 1.7 Regulation of Ornithine decarboxylase and its antizyme

Experimental data show that residues 117-145 of mammalian ODC are recognized by antizyme. So, it is called as Antizyme Binding Element (AzBE). But only AzBE region is not sufficient for degradation of ODC. There are other structural elements present within ODC which are responsible for rapid degradation of ODC. In mammals, when antizyme and ODC come together there is a conformational change in ODC which results in exposure of the C-terminus of ODC (Li and Coffino, 1993). The unstructured C-terminal region pulls whole ODC towards proteasome for degradation. Actually proteasome begins degradation of ODC at the C-terminus (Zhang et al., 2004). This region is absent in *T. brucei* ODC, which makes the protein more stable (Seely et

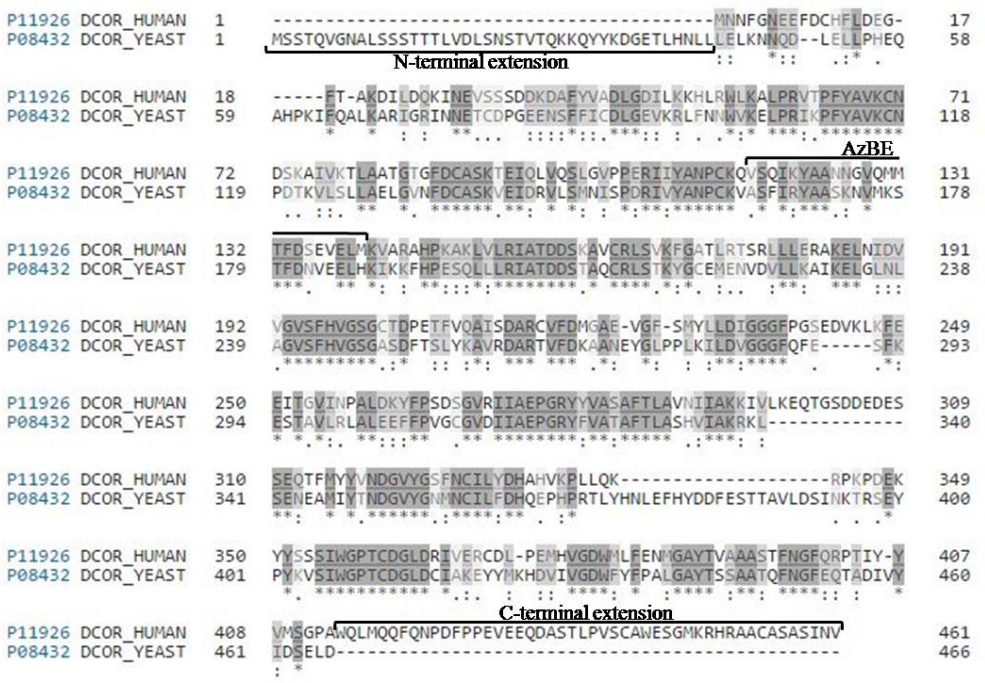
al., 1985). Deletion of the 37 residues at the C-terminal part of mammalian ODC stabilizes the protein even in the presence of antizyme. Attachment of only the C-terminal residues of mouse ODC to other stable proteins caused their degradation via proteasome (Zhang et al., 2003). Mutation of Cys441, which is located in this sequence, stabilized ODC even in the presence of excess antizyme (Matsufuji et al., 1995). This C-terminal region is disordered in human ODC crystal structure (Almrud et al., 2000; Kern et al., 1999). These observations imply that the last few amino acids of ODC are critical for proteasomal degradation of the protein. The C-terminal half of antizyme interacts with ODC. And the interaction is enough to expose the C-terminus of ODC. Antizyme increases accessibility of the C- terminus of ODC by changing conformation of ODC and exposed C- terminus is recognized by proteasomes.

1.3.3 Comparison of mammalian and yeast ornithine decarboxylase

When mammalian and yeast ODCs are aligned, it shows 40% sequence homology (Fig. 1.8A). Mammalian ODC (mODC) has an extended C-terminus, which is not present in yODC. On the other hand, yODC contains an N- terminal extension not found in the mammalian ODC (Fig. 1.8B). From sequence alignment it is found that AzBE correspond to the residues 164-187 of yODC and 117-140 in mODC. In the mODC one more degradation signal is present in its C-terminus (Ghoda et al., 1990; Ghoda et al., 1989), while N- terminus of yODC is found to be required for its rapid degradation (Gandre et al., 2002). Although the mammalian ODC degradation signal is recognized by both mammalian and yeast proteasomes, yODC is stable in mammalian cells (Gandre et al., 2002; Porat et al., 2008), suggesting that yeast ODC degradation signal is specific for yeast proteasomes. N-terminal domain of yeast ODC is essential for antizyme dependent degradation. In *S. cerevisiae* a deletion of 47 residues from the

ODC N-terminus led to accumulation of the protein in wild-type cells to levels similar to those in yeast antizyme (*oaz1*) deleted cells (Godderz et al., 2011; Palanimurugan et al., 2004).

(A)



(B)

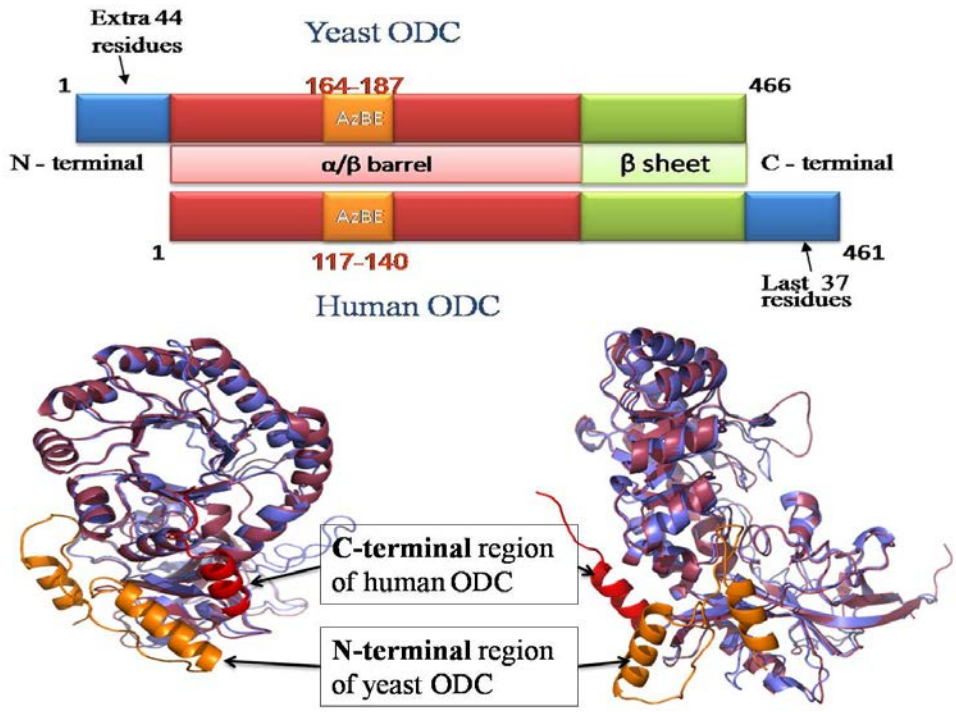


Figure 1.8 (A) Pairwise sequence alignment of human and yeast ODC (uniprot) (B) Structural comparison of yeast ODC and human ODC

1.4 Antizyme, a competitor of ubiquitin

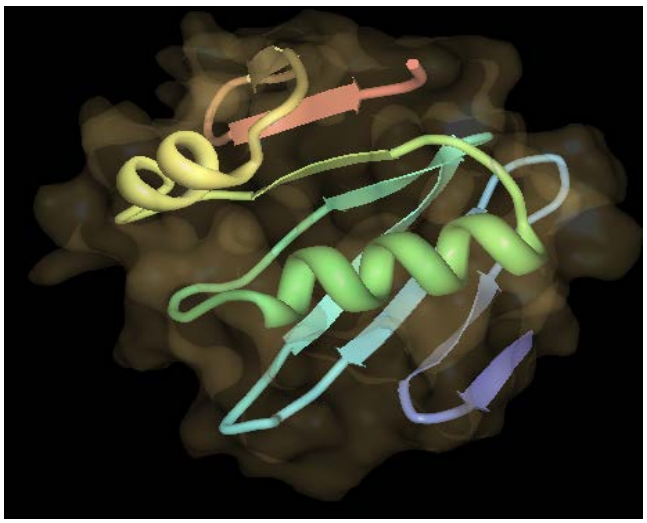
Antizyme is unusual tag of protein degradation through proteasome. Majority of proteasome substrates are degraded by polyubiquitinylation process. In other words ubiquitin is a typical tag for specific protein degradation via proteasome. Antizyme is functional homologue of ubiquitin. There are many proteins in the cell which are degraded by antizyme mediated mechanism. Examples include ornithine decarboxylase (Bercovich et al., 1989; Murakami et al., 1992; Rosenberg-Hasson et al., 1989), p21/Cip1 (Sheaff et al., 2000), TCR*α* (Yu et al., 1997), I*k*B*a* (Krappmann et al., 1996), c-Jun (Jariel-Encontre et al., 1995), calmodulin (Tarcsa et al., 2000) and thymidylate synthase (Forsthoefel et al., 2004). However, antizyme the mediator of ubiquitin independent degradation pathway is itself degraded by ubiquitination (Gandre et al., 2002).

Antizyme was first known as an inhibitor of the polyamine inducible enzyme ornithine decarboxylase in rat liver (Heller et al., 1976). Antizyme is an unusual protein in several aspects. It is known to catalyze the ATP-dependent but ubiquitin independent degradation of ODC by the 26S proteasome (Hayashi et al., 1996; Murakami et al., 1992). In mammals, several isoforms of antizyme are identified known as AZ1, AZ2, AZ3, AZ4 (Heller et al., 1976; Ivanov et al., 1998; Ivanov et al., 2000a; Ivanov et al., 2000b). All four isoforms of antizyme interact with ODC but with different biological consequences. Among those, AZ1 disrupts enzymatically active ODC homodimers by forming ODC/AZ heterodimers. AZ1 is ubiquitously expressed. It inhibits ODC activity and promotes ODC for degradation. AZ2 is expressed similar to AZ1 but expression level is low compared to AZ1. AZ2 is more conserved evolutionarily than AZ1. AZ2 is able to inhibit ODC activity but failed to degrade ODC. AZ3 inhibits polyamine uptake and ODC activity, but does not stimulate ODC degradation (Snapir et al., 2009). The

expression and molecular functions of AZ3 appears to be limited to the testis and AZ4 has not been analyzed in detail. Orthologue of mammalian antizyme was identified in budding yeast (*Saccharomyces cerevisiae*) (Gupta et al., 2001; Palanimurugan et al., 2004). There is only one type of antizyme (*OAzI*) in yeast. AZ1 is functionally homologous to yeast antizyme (OAz1) confirmed by sequence analysis.

1.4.1 Structure of antizyme

The full length rat antizyme-1 (AZ1) carries 227 amino acids and its molecular weight is 26.5 kDa. The NMR structure of rat AZ1fragment corresponding to amino acid residues from 87-227 has been solved. It contains eight β strands and two α helices. The β-strands form a mixed parallel and antiparallel β-sheet like structure (Fig. 1.9A). At the level of primary sequence, antizyme is not showing similarity with any protein of known structure. So, antizyme exhibits a novel arrangement of its strands and helices (Hoffman et al., 2005). Sequence alignment of yeast and human antizyme shows yeast antizyme contains more insertions (Fig. 1.9B).



(A)

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P54370 OAZ1_RAT   1    ---MVK---SSLQRILNSHCFAREKEGDKRSATLHASRTMPLLSQHSRGGCSSESSRVAL   54
Q02803 OAZ_YEAST  1    MYEVIQKRKTKIINVLQSPELMRLIED---PSNLGISLHFPVSSLLKSNKCTPMPKLS--   55
                          :::   :.: .:*:*  : *  *     :.*  *  :*: *  .   *:   .

P54370 OAZ1_RAT   55   HCCSNLGPGPRWCSDVPHPPLKIPGGRGNSQRDHSLSASILYSDERL----NVTEEPTSN  110
Q02803 OAZ_YEAST  56   TYSLASGGFKDWCADIPLDV---PPEIDIID--FYW-DVILCMESQFILDYNVPSKNKGN  109
                        .   *    **:*:*      *      :  .     **  :.::    ** .: ..*

P54370 OAZ1_RAT   111  DKTRVL---------SIQCTL---------------TEAKQVTWRAVWNGG-GLYIE---  142
Q02803 OAZ_YEAST  110  NQKSVAKLLKNKLVNDMKTTLKRLIYNENTKQYKNNNSHDGYNWRKLGSQYFILYLPLFT  169
                       ::. *          .:: **                .. .  .** : .    **:

P54370 OAZ1_RAT   143  ----------------LPAGPLPEG---------SKDSFAALLEFAEEQLRAD-------  170
Q02803 OAZ_YEAST  170  QELIWCKLNENYFHVVLPSLLNSRNVHDNHSTYINKDWLLALLELTSN-LNQNFKFEYMK  228
                                    **:    .          .** : ****::.: *. :

P54370 OAZ1_RAT   171  -HVFICFPKNREDRAALLRTFSFLGFEIVRPGHPLVP-------------KRPDACFMVY  216
Q02803 OAZ_YEAST  229  LRLYILRDDLINNGLDLLKNLNWVGGKLIKNEDREVLLNSTDLATDSISHLLGDENFVIL  288
                        :::*   .  ::   **:.:.::* ::::  .  *                  *  *::

P54370 OAZ1_RAT   217  TLEREDPGEED                                                  227
Q02803 OAZ_YEAST  289  EFEC-------                                                  292
                        :*
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(B)

Figure 1.9 (A) NMR structure of rat antizyme fragment (87 to 227 residues) (PDB ID: 1ZO0) (B) Pairwise sequence alignment of rat and yeast antizyme

1.4.2 Function and regulation of antizyme

Antizyme is a central component of an autoregulatory circuit of polyamine biosynthesis pathway. The polyamines (putrescine, spermidine, and spermine) are abundant multivalent organic cations. They are mainly bound to negatively charged molecules such as DNA and RNA. Polyamines are not only essential for normal cell growth and differentiation but also play an important role in cell proliferation. Over accumulation of polyamines leads to development of cancer. Polyamines are found in prokaryotes and eukaryotes.

Synthesis of antizyme is regulated by polyamine concentration in the cells. Actually, a complete antizyme protein is encoded by two different overlapping open reading frames (ORFs) of mRNA. The first (ORF1) is short and has start codon (AUG) and stop codon (UGA). Translation is initiated with ORF1, but is terminated immediately. The second (ORF2) encodes most of the protein, but lacks initiation codon. The synthesis of a complete antizyme protein requires both the ORFs. ORF2 can

be translated only by starting with ORF1. At this point, high level of polyamine stimulates +1 ribosomal frameshifting at the end of ORF1. Frameshifting by one base just before the stop codon of ORF1 continues translation into the +1 frame to the end of ORF2 (Fig.1.10) (Matsufuji et al., 1995). This in turn increases antizyme levels in the cell. This antizyme forms ODC/AZ heterodimers and in turn inhibits formation of ODC/ODC homodimer. In the heterodimer, as mentioned earlier ODC degradation signal is exposed which is recognized by the proteasome leading to degradation of ODC and recycling of antizyme. The process of translation of antizyme mRNA by +1 ribosomal frameshifting and its signal (5' UCC UGA U 3') are highly conserved in almost all known antizyme genes (Ivanov et al., 2004).

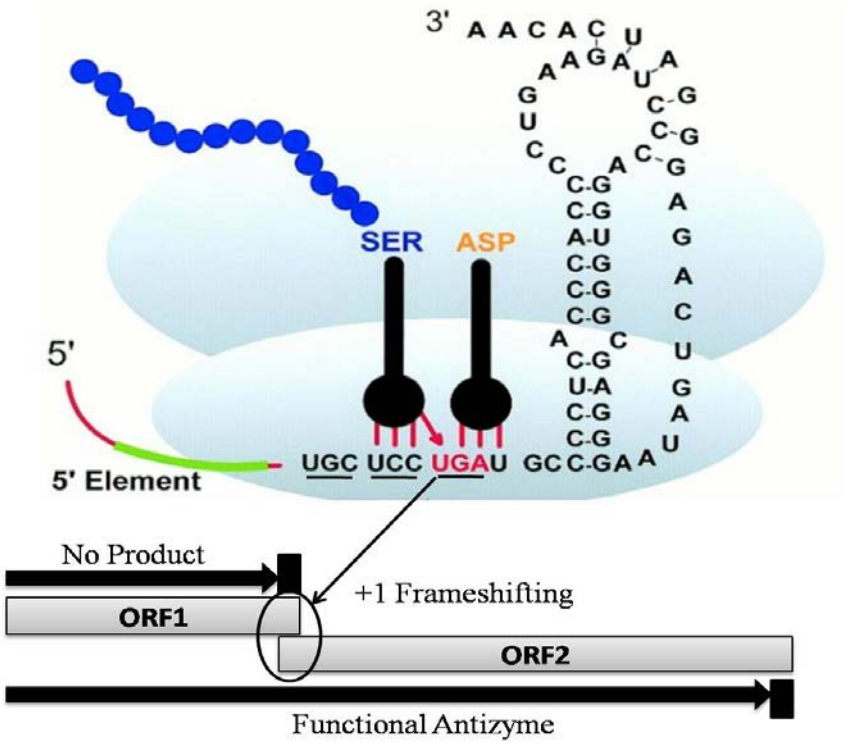

Figure 1.10 The mechanism of +1 Frmaeshifting for synthesis full length antizyme

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1.5 Why ODC has special mechanisms for its regulation?

It is very difficult to justify this question because the basic criteria of nature for ubiquitin dependent and independent mechanism for degradation remain unknown. One possible explanation may answer this query. ODC is key regulatory enzyme in polyamine biosynthesis pathway. Polyamines are having functionally great importance in intracellular process. Since polyamines participate in various cellular functions in the nucleus, mitochondria, plasma membrane, secretory vesicles and cytoplasm. ODC has extremely short half-life and thus minute amount of the protein is present in the cell. Investigations on the localization of ODC have proven difficult due to its extremely short half-life. Because of its important role in polyamine biosynthesis, nature evolved special regulation mechanisms for ODC.

1.6 ODC: Antizyme interface

The interaction between ODC and antizyme is important for regulating the levels of polyamines in the cells. Hence, to what extent the two proteins have coevolved to maintain their interaction from the lower most eukaryotes to the highly evolved?

- Antizyme binding element (AzBE) on ODC (residues 117–140) was shown to contain an electropositive patch. It is partially buried in the ODC dimer as seen in the crystal structure. But it is proposed to be solvent accessible in the monomeric form which binds Antizyme (Almrud et al., 2000). On the other hand, the NMR solution structure of antizyme, reveals that the patch of negatively charged residues is important for binding to ODC. It is also known that only the C-terminal half of Az is important for binding and inhibition of enzymatic activity of ODC (Ichiba et al., 1994). But only C-terminal is not sufficient to promote degradation of ODC. N-

terminal region of antizyme (55-212 amino acids) directs ODC for degradation (Li and Coffino, 1994).

- Site-directed mutagenesis of ODC and Az, followed by double mutant cycle experiments have given information about ODC-Az interface (Cohavi et al., 2009). In this study they have shown interacting residues of mouse ODC: AZ (ODCF397–H202Az, F397–F173, F397–E196, Y323–F173, Y323–I197, K92–E219, K141–D154) (Fig. 1.11).

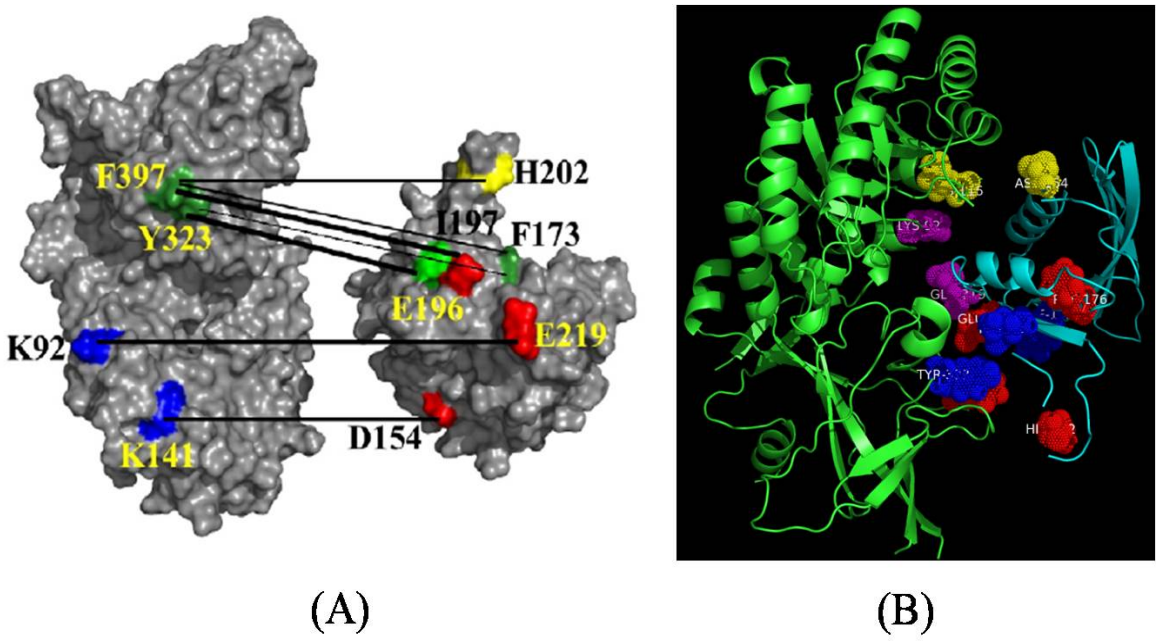

Figure 1.11 Double mutant cycle analysis of ODC: Az interface

(A) ODC/AZ interface from double mutant cycle analysis. Lines connect the interacting residues. (Reprinted from Publication (Cohavi et al., 2009) Copyright (2009), with permission from Elsevier)

(B) Same DMC model in PyMol view: Showing interacting residues on ODC and Antizyme.

1.7 ODC as a tool for targeted protein degradation

Antizyme, delivers ODC to proteasome for degradation. But only antizyme is not sufficient for ODC degradation. Some degradation signals lie within the ODC, which make it a favourable substrate for proteasome. It has been reported that the ODC/AZ system is more effective at achieving targeted protein degradation than other types of degradation (Matsuzawa et al., 2005). Advantage of ODC/AZ is that posttranslational modification of the target protein (unlike ubiquitination) is not required, thus providing a more direct means of delivering ligand target complexes to the proteasome for degradation (Fig. 1.12). In an earlier study, several peptides and proteins expressed as chimeric extensions of ODC have been shown to undergo targeted protein degradation (table 1.1). As mentioned earlier the C-terminal residues of mODC and N-terminal 44-50 residues of yODC can act as a transplantable degrons without rest of the protein (Joshi et al., 2015).

Table 1.1: Comparison of degrons efficiency for targeted protein degradation (Matsuzawa et al., 2005)

Ligand	Target	ODC (N)	ODC plus AZ	SIP (N)	Siah (N)	E7 (C)	Fbx7 (C)	Ub1 (N)	Ub4 (N)	S5a (N)
TRAF6-C	TRAF6	↓	↓	–	–	–	–	–	–	–
RANK-pep.	TRAF6	–	↓	–	–	–	–	–	–	–
CD40CT	TRAF2	–	–	nd	nd	nd	nd	nd	nd	–
I-TRAF	TRAF2	–	–	–	nd	nd	nd	nd	nd	–
IKKα(LZ)	IKKα	–	–	–	nd	nd	nd	nd	nd	–
IKKβ(LZ)	IKKβ	↓	↓	–	nd	nd	nd	nd	nd	–
E7	Rb	–	↓	–	–	nd	–	–	–	–
Caspase9(CARD)	Apaf1	–	–	–	–	–	–	–	–	nd
Apaf1(CARD)	Caspase9	nd	–	–	–	–	–	–	–	nd
FADD(DED)	Caspase8	nd	–	–	–	–	–	–	–	nd
BAG-1	HSP70	–	–	nd	nd	nd	nd	nd	nd	nd
p21	Cdk2	↓	↓	nd	–	–	nd	nd	nd	nd
Success ratio	All targets	3/12	5/12	0/9	0/7	0/6	0/6	0/6	0/6	0/7

N, N-terminal fusion; C, C-terminal fusion; nd, not done; ↓, decrease; –, no change.

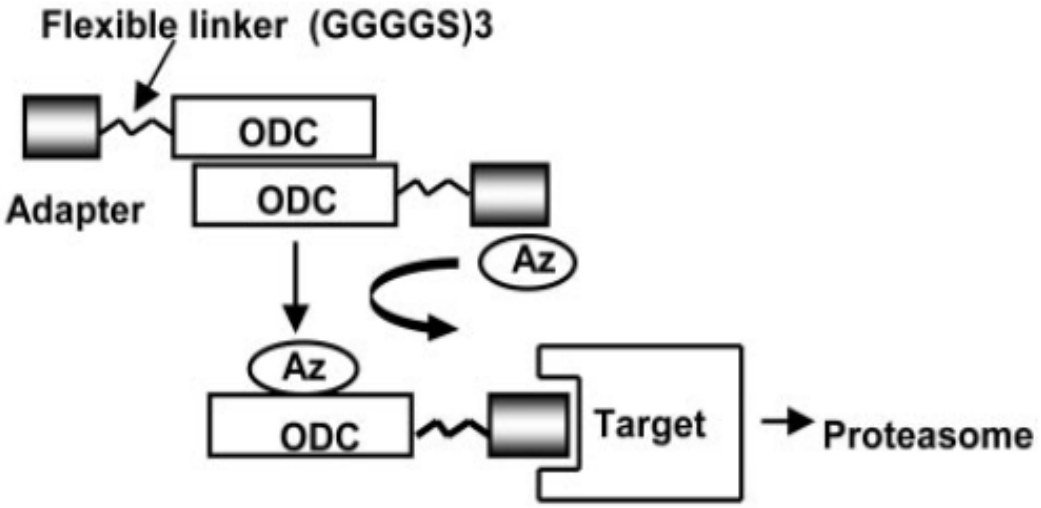

Figure 1.12 ODC chimeric fusion as a vehicle for targeted protein degradation (Matsuzawa et al., 2005)

1.8 Hypothesis of the present study

Earlier targeted protein degradation was demonstrated successfully using chimeric constructs of human ODC grafted on protein interacting domains. Two main shortcomings encountered with the use of full length ODC are its enormous size and elevated enzyme activity due to over expression of the protein. In the present study it was decided to engineer the degrons of ODC as efficient vehicle for targeted protein degradation, keeping the degrons intact and pruning ODC to minimal size.

In order to test this hypothesis degrons of different sizes were designed from the sequences of yeast and mammalian ODCs i.e. four degrons from yeast ODC and one from mouse ODC. To achieve efficient targeted protein degradation, the degrons of yeast and mouse ODCs were used alone and in combinations (Fig. 1.13 & table 1.2). Degrons of ODCs were tagged to different reporter proteins at N and C terminal

and both and examined stability of reporter protein. Simultaneously structural characterization of selected degrons was carried out. List of the selected degrons are mentioned in Table 1.2.

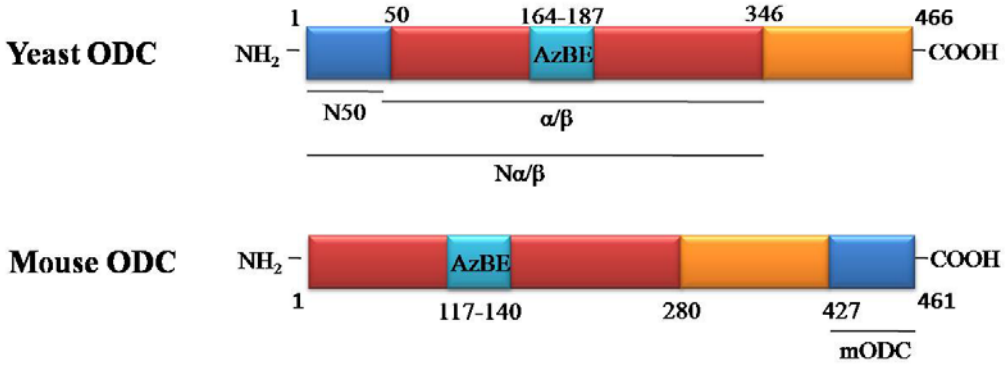

Figure 1.13 Schematic representation of degrons position on yeast and mouse ODCs

Table 1.2: List of degrons used in this study

Degrons	Position in ODC sequence	Details
N50	1-50	First 50 residues of yODC
α/β	51-346	α/β barrel domain of yODC
Nα/β	1-346	Combination of N50 + α/β domain (N-terminal domain of yODC)
AzBE	149-208	Antizyme Binding Element
mODC	427-461	Last 37 residues of mouse ODC
α/β-mODC	51-346 (yODC) 427-461 (mODC)	α/β barral domain combine with mODC

AzBE-mODC	149-208 (yODC) 427-461 (mODC)	Antizyme Binding Element followed by mODC
N50-mODC	1-50 (yODC) 427-461 (mODC)	Extra first 50 residues of yODC with last 37 residues of mODC
Nα/β-mODC	1-346 (yODC) 427-461 (mODC)	N-terminal domain of yeast ODC attached with last 37 residues of mODC

1.9 Major objectives of the present study

1) Structural characterization of degradation determinant signals (degrons) of yeast and mouse ornithine decarboxylase, in isolation from rest of the protein sequences and in combination with other degrons.

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

3) Studies on the coevolution of ornithine decarboxylase and antizyme.

This thesis is divided in four major chapters:

Chapter 1 (current chapter) gives general introduction to protein degradation in eukaryotes, the process of targeted protein degradation and the strategies developed for artificially inducing targeted protein degradation. Degradation of Ornithine Decarboxylase (ODC), role of antizyme, ODC antizyme interaction, the concept of degrons and hypothesis of the study are presented here.

Chapter 2 is focused on structural characterization of degrons of yeast and mouse ODC alone and in combination. This chapter describes in detail about the cloning of DNA fragments corresponding to degrons in *Escherichia coli*, peptide expression, purification and structural studies. Main aim of this chapter is to know structure of degrons without rest of the protein. Secondary structure analysis was carried out by circular dichroism and fluorescence spectroscopy was used to know tertiary structure of the peptides.

Chapter 3 deals with functional characterization of degrons of yeast and mouse ODCs. This chapter describes degradation potential of degrons of ODC for targeted protein degradation. Degrons of ODC are tagged to three reporter proteins (yEGFP, URA3 and β-galactosidase) at N terminal, C terminal and both N and C terminals. The degradation potentials of degrons were also compared in the presence and absence of antizyme induced by polyamines.

Chapter 4 describes studies on the coevolution of ornithine decarboxylase and antizyme protein. In this part of the work, sequences of ODC and antizyme were collected from several organisms and performed multiple sequence alignment. Distance matrices for ODC and antizyme were generated by Dayhoff method. Correlation coefficient was calculated for these two matrices.