# FUNCTIONAL AND BIOCHEMICAL ANALYSIS OF A NOVEL DEUBIQUITINATING ENZYME, USP32

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## ABSTRACT

### FUNCTIONAL AND BIOCHEMICAL ANALYSIS OF A NOVEL DEUBIQUITINATING ENZYME, USP32

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Ubiquitylation is an important post-translational modification and can be reversed by the action of deubiquitinating (DUB) enzymes. The ubiquitylation and deubiquitylation of target proteins are significant in terms of regulating cellular events such as protein degradation, signal transduction, vesicle trafficking, DNA repair and apoptosis. Chromosomal band 17q23 is frequently amplified in breast cancers and harbors a predicted ubiquitin specific protease gene, USP32 (ubiquitin specific protease 32). Given its potential role in breast cancer, we aimed to characterize USP32 for its potential DUB activity. Bioinformatic analysis of USP32 and known yeast and mouse DUBs suggested presence of Cys-His domains which are common in active DUBs of the USP superfamily. Our in vivo and in vitro DUB activity assays revealed that USP32 was indeed an active deubiquitinating enzyme. To investigate its substrate specificity and kinetic properties, USP32 was expressed in insect cell culture to be isolated and purified. Using isolated USP32 protein, diubiquitin assay was performed with all seven types of diubiquitin (K6, K11, K27, K29, K33, K48 and K63) as well as linear diubiquitin. Results showed that USP32 was able to cleave all seven types of ubiquitin linkages with higher cleavage efficiency for K6, K11, K48 and K63-linked diubiquitin. Moreover, kinetic

parameters, $K_m$, $k_{cat}$ and $k_{cat/}$ $K_m$, suggested that full length protein had lower affinity for potential substrates and lower catalytic activity compared to the catalytic domain alone. These data suggested the importance of USP32 tertiary structure and possible role of other non DUB domains (e.g. EF hand domain) which may be regulated by an as of unknown mechanism in cells. Further investigations are underway to understand the functions of USP32 in cells and how it may contribute to breast tumorigenesis.

Key Words: Ubiquitin, deubiquitylation, DUBs, ubiquitin specific proteases, Ub chains, USP32.

# ÖZ

## YENİ BİR DEUBİKİTİNAZ ENZİMİ OLAN USP32' NİN FONKSİYONEL VE BİYOKİMYASAL ANALİZİ

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Ubikuitinasyon hücre içerisinde önemli bir tranlasyon sonrası modifikasyondur ve deubikitinaz enzimleri (DUBs) tarafından geri çevirilebilir. Proteinlerin ubikuitinasyon ve deubikuitinasyonu hücre içerisinde proteinlerin yıkımı, sinyal iletimi, vesikül trafiği, DNA tamiri ve apoptoz gibi birçok hücresel olayın düzenlemesinde görev alır. USP32, ubiquitin specific proteaz 32, meme kanserinde sıklıkla amplikasyona uğrayan 17q23 kromozom bölgeside yer alan bir gendir. Çalışmamızda USP32'nin meme kanserindeki potansiyel rolünü anlayabilmek için USP32 proteininin deubikuitinaz aktivitesini karakterize etmek amaçlandı. Biyoinformatik analizler USP32'nin ubikitin spesifik proteaz enzim ailesinde yaygın olarak bulunan Sis ve His domainlerine sahip olduğunu öngördü. *In vivo* ve *in vitro* DUB aktivite deneyleri USP32'nin aktif bir deubikuitinaz enzimi olduğunu ortaya koydu. USP32'nin aktif bir DUB enzimi olduğu belirlendikten sonra substrat özgüllüğü ve kinetik özelliklerinin incelenmek için USP32 proteini sinek hücre kültüründe ifade edildi ve izole edildi. USP32'nin substrat özgüllüğünün ve hücre içerisindeki muhtemel rollerinin anlaşılabilmesi için birbirlerine linear ve 7 farklı lizin grubu ile bağlanmış diubikuitinler (K6, K11, K27, K29, K33, K48 and K63) kullanılarak diubikuitin deneyi yapıldı. Sonuçlar USP32'nin 7 farklı diukuitin'in

hepsini tanıyıp kestiğini ve bununla birlikte K6, K11, K48 ve K63 daha yüksek verimlilikle kesebildiğini gösterdi. Kinetik parametreler, Km, kcat ve kcat / Km, tam uzunlukta proteinin katalitik domain proteinine göre potansiyel subsratına karşı daha düşük afinitesi olduğunu ve daha düşük katalitik reaksiyon verimine sahip olduğunu göstermiştir. Bu bulgular da USP32 nin üçüncül yapısının ve proteaz aktivitesi olmayan diğer domainlerinin önemine işaret etmektedir, Hücre içerisinde USP32 fonksiyonları ve meme tümörünün gelişimine nasıl katkıda bulunabileceğini anlamak için çalışmalar devam etmektedir.

Anahtar Kelimeler: Ubikuitin, deubikuitinasyon, DUBs, ubiquitin specific proteases, Ub zinciri, USP32.

To my brother

TAMER SAPMAZ

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| | |
|---|---|
| aa | Amino acid |
| ATP | Adenosine triphosphate |
| bp | Base pair |
| CDS | Coding sequence |
| dNTP | Deoxyribonucleotide triphosphate |
| DUBs | Deubiquitinating enzymes |
| FP | Fluorescence polarization |
| GST | Glutathione S-transferases |
| IPTG | Isopropyl β-D-1-thiogalactopyranoside |
| K | Lysine |
| kDa | Kilodalton |
| LB | Luria Bertani |
| nt | Nucleotide |
| PBS | Phosphate Buffered Saline |
| Rho | Rhodamine |
| SDS | Sodium Dodecyl Sulfate |
| TAE | Tris Acetate EDTA |

| | |
|---|---|
| TBE | Tris Borate EDTA |
| Ub | Ubiquitin |
| UBL | Ubiquitin Like Modifier |
| USP | Ubiquitin Specific Protease |

# CHAPTER I

# INTRODUCTION

## 1.1. Ubiquitylation

Ubiquitin (Ubiquitous immunopoietic polypeptide) was first discovered in 1975 as a 8.5 kDa conserved polypeptide [1]. Ubiquitin (Ub) is a small 76 amino acid polypeptide which is an important component of the ATP-dependent proteolytic system in eukaryotic cells [2]. Ubiquitin is covalently attached to target proteins to regulate their functions and lifespan. There are three different enzymes, E1 (Ubiquitin activating enzyme), E2 (Ubiquitin conjugating enzyme) and E3 (Ubiquitin ligase), by which ubiquitylation of target protein is processed and ubiquitin linkage types are determined [2, 3].

In this process, E1 binds to ATP and ubiquitin to catalyze the adenylation of C-terminus of Ub. Therefore, a covalent thioester bond occurs between adenylated Ub and E1 catalytic cysteine site. Then, Ub is transferred to an E2 enzyme after activation of Ub in an ATP-dependent manner. E2-Ub complex interacts with the E3 enzyme which transfers Ub to specific lysine groups of substrate proteins via isopeptide bonds. This isopeptide bond generally forms between C-terminus glycine group (Gly76) and ε-amino group of a lysine group of substrate. On the other hand, previous studies showed that Ub can also modify amino terminus [4, 5], serine hydroxyl [6] and cysteine thiol groups [7] of its substrate protein [8].

In human genome, there are only 2 known E1 ubiquitin-activating enzymes, approximately 40 E2 ubiquitin-conjugating enzymes and more than 1000 E3 ligase enzymes [9].

E3 ligase enzymes harbor HECT, RING or U-box domains. E3 enzymes, containing RING or U-box domain, can directly transfer Ub from E2 enzyme to the substrate protein. On the other hand, HECT domain containing E3s catalyze the transfer of Ub from E2 to their catalytic cysteine sites and then transfer it to the substrate. Ubiquitinylation processing and different mechanisms of E3 ligase are summarized in Figure 1.1.

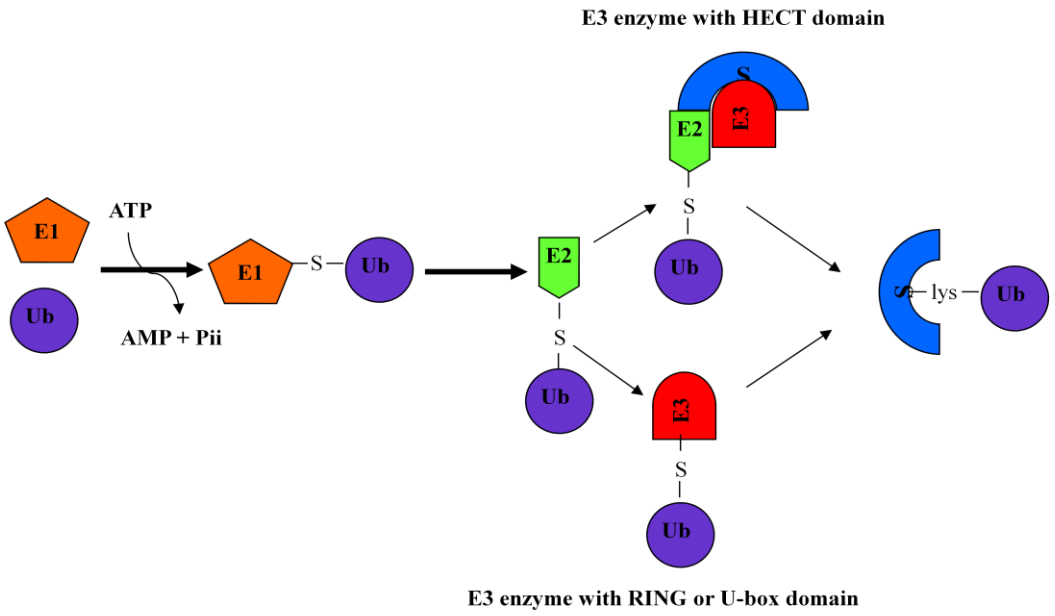



**Figure 1.1.** Schematic view of ubiquitylation process. The conjugation of ubiquitin to target protein is processed by the E1 ubiquitin-activating enzyme, E2 ubiquitin-conjugating enzyme and E3 ligase. E3 ligase enzymes include HECT, RING or U-box domains. E3 ligase directly (RING or U-box) or (HECT) transfer Ub to target protein, depending on type of domain.

### 1.2. Types of ubiquitin chains and their functions

Target proteins can be modified by monoubiquitylation, multi-monoubiquitylation or polyubiquitylation. Polyubiquitin chains are formed by different lysine residues of ubiquitin. Ubiquitin contains seven lysine residues at 6, 11, 27, 29, 33, 48 and 63 amino acid positions (K6, K11, K27, K29, K33, K48 and K63). All seven lysine

residues which [10] are important for formation of ubiquitin chains. C terminus of one ubiquitin moiety is conjugated to another ubiquitin moiety using these seven lysine groups or its N-terminal glycine group (linear linkage) to create polyubiquitin chains. These different linkage types cause different conformations of ubiquitin chains. Recent studies demonstrated that K29-, K33- [11], linear- (called as Met1-linked) [12] and K63 [13] -linkages encourage an extended conformation while K6, K11, K27 [11] and K48- [14] linked chains promote closed conformation for ubiquitin chain structure (Figure 1.2).

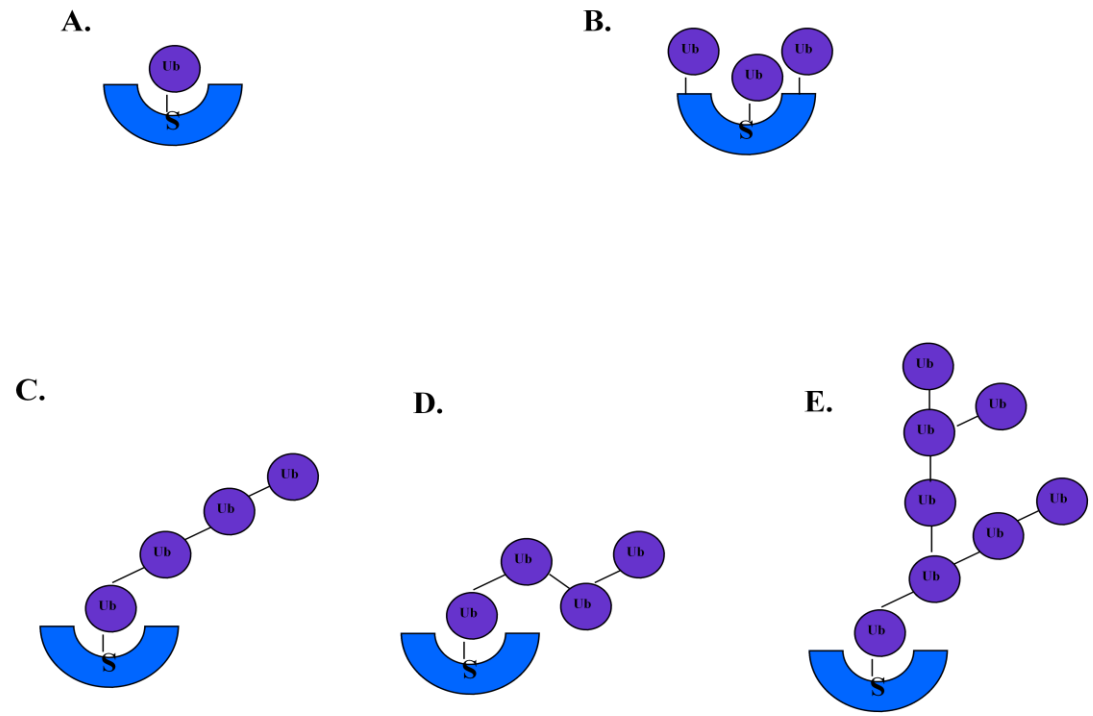



**Figure 1.2.** Different conformations of ubiquitin chains. **S** indicates target protein (Substrate). Substrate can be conjugated to ubiquitin by different types of linkages including monoubiquitylation **(A)**, multi-monoubiquitylation **(B)** and polyubiquitylation **(C, D, and E)**. **C.** Extended conformation of ubiquitin chain, formed by K29, K33, K63 and Met1. **D.** Closed conformation of ubiquitin chain, formed by K6, K11, K27 and K48. **E.** Mixed or branched linkage conformation of ubiquitin chain.

E2 and E3 enzymes play an important role in ubiquitin linkage types and substrate specifity. The members of E2 enzyme family contain highly conserved catalytic

domains with 150-200 amino acid residues which provide a surface to interact with ubiquitin and E3 enzymes.

Previous studies performed in yeast and mammalian cells have shown that E2 enzymes are essential factors for formation of ubiquitin linkage types. UBE2N-UBE2V1 E2 complex (Mms2 (methyl methanesulfonate sensitive 2)-Ubc13 E2 complex in yeast) enzyme promote the formation of K63 specific chain [15, 16] whereas Cdc34 (cell division cycle 34 homolog) catalyze K48 specific chain formation [17]. Furthermore, a recent study showed that another E2 ubiquitin conjugating enzyme, UBE2S, is able to elongate K11 specific chain [18].

E3 ligases are also important determining factors in addition to E2 conjugating enzymes. Types of ubiquitin linkages are determined by the enzyme carrying Ub with thioester bond like E2 enzymes and E3-HECT ligase. E3-RING enzymes are not responsible for determination of Ub-linkage because they do not have active cysteine site in order to bind to Ub. E2 enzymes provide elongation of specific Ub chains in E3-RING-depended Ub-processing. Previous studies demonstrated that E6AP E3-HECT ligase creates K48 specific Ub chain [19, 20] while Rsp5 E3-HECT ligase is responsible for generating K63 specific Ub-linkages [21].

Protein ubiquitylation, an important type of post-translational modification, play roles in vital cellular events including cell-cycle regulation, signal transduction, DNA repair, vesicle transport, etc. Types of polyubiquitin chains that are conjugated to specific substrates determine which cellular processes are regulated by ubiquitylation. Previous studies showed that cell cycle progression and protein degradation processes are abolished when lysine 48 (K48) residue is changed to an arginine (R). This suggested that K48-linked polyubiquitin chain targets the proteosomal degradation pathway [22]. K63 linkage is important for DNA repair [23], vesicle trafficking and kinase activation (signal transduction) [24]. Furthermore, K11-linked poylubiquitin chain is associated with endoplasmic reticulum-associated degradation (ERAD) mechanism [25] and proteosomal

degradation [26]. All cellular functions regulated by different types of Ub linkages are summarized in Figure 1.3.

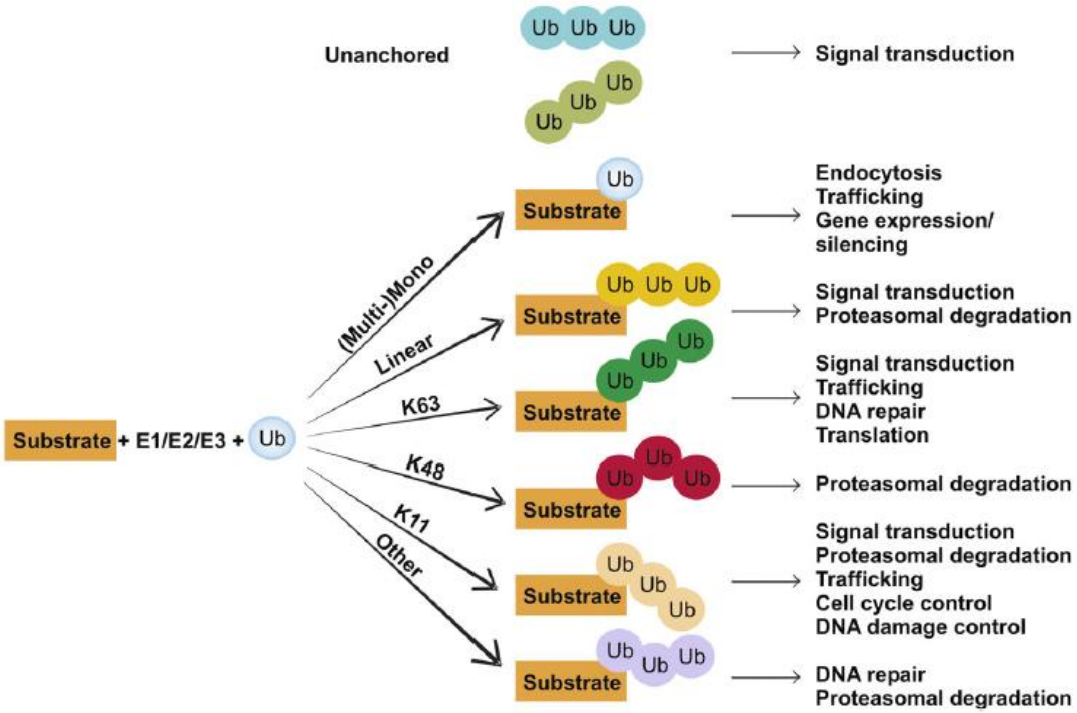



**Figure 1.3.** Summary of different types of ubiquitin linkage and their functional roles in cellular events. Each type of Ub-linkage plays roles in different cellular events, including vesicle trafficking, signal transduction, DNA repair, DNA damage control, proteosomal degradation, etc. Even unanchored ubiquitin plays a role in signal transduction. Taken from [27].

### 1.3. Deubiquitylation and deubiquitinating enzymes

Ubiquitylation is a reversible process in which ubiquitin is removed from target proteins by means of a specific enzyme group known as **deubiquitinating enzymes (DUBs)**. More than 90 DUBs are found in the human genome [28]. These enzymes are classified into two main classes named as cysteine proteases and

metalloproteases. Four of the DUB families belong to cysteine proteases class. One DUB family is categorized as metalloproteases.

### 1.3.1. Cysteine Protease DUBs

Cysteine Protease DUBs include four groups of DUBs namely, Ovarian Tumour Proteases (OTUs), Ubiquitin C-terminal Hydrolases (UCHs), Machado-Josephin Disease proteases (MJDs) and Ubiquitin Specific Proteases (USPs). All groups belonging to cysteine proteases possess common architecture and mechanism to remove ubiquitin from target substrates [29]. These groups of deubiquitinating enzymes contain cysteine residue in their active sites. The thiol group of cysteine residue performs nucleophilic attack to the carbonyl carbon of isopeptide bond between ubiquitin and lysine group of target protein. Nucleophilic attack leads to the removal of target protein and then ubiquitin is released from the DUB enzyme by hydrolysis (Figure1.4) [30].

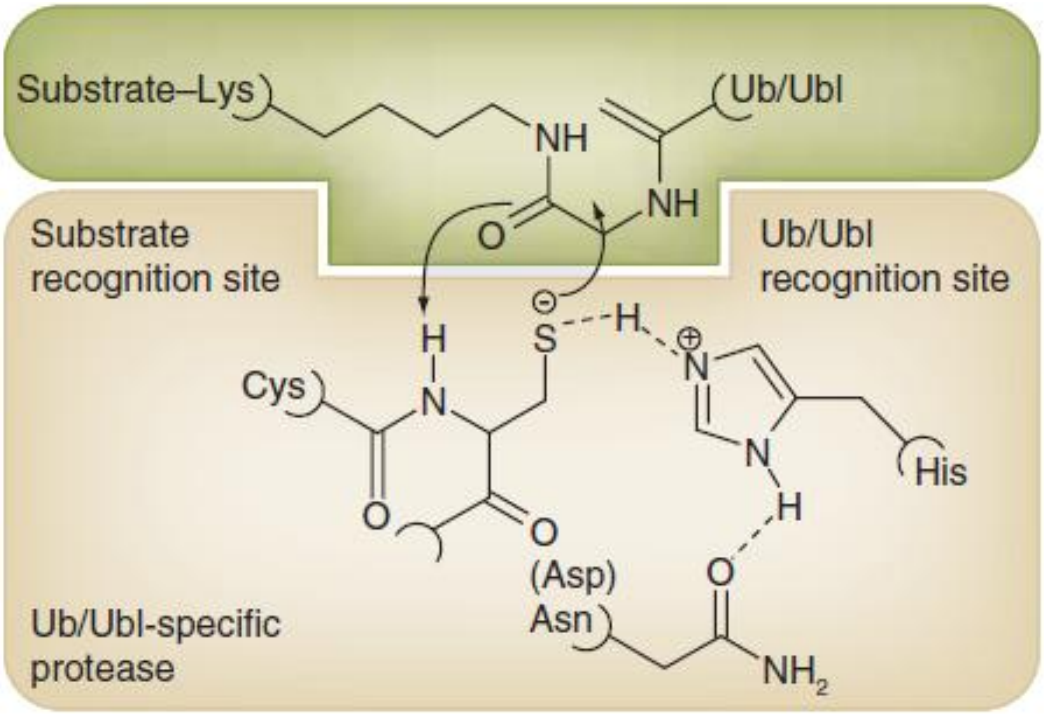



**Figure 1.4.** Basic biochemical mechanism of removal of Ubiquitin from target protein substrate. Cystein residue of DUB active site attacks to carbonyl carbon of

isopeptide bond between lysine group of target protein and C-terminus of Ub. This process is facilitated by histidine and aspartate/asparagine residue of the DUB active site. Ubiquitin is released from enzyme by hydrolysis. Ub:Ubiquitin and Ubl: Ubiquitin-Like Modifier. Figure is taken from [30].

#### 1.3.1.1. Ovarian tumour (OTU) superfamily

The first member of this family was identified in *Drosophila Melanogaster* with high homology to plant, human and viruses [31]. Then, a number of members of OTU family were experimentally described as deubiquitinating enzymes [32]. OTU superfamily has 15 members and is divided into 3 subclasses namely, **otubains** (OTUB1 and OTUB2), **A20-like OTUs** (A20/TNFAIP3; Cezanne, Cezanne 2, TRABID and VCPIP1) and **OTUDs** (OTUD1, OTUD2/YOD1, OTUD3, OTUD4, OTUD5, OTUD6A, OTUD6B and ALG13) [33]. The family members play a role in cell signaling pathways such as, NF-κB signaling [34], Wnt signaling [35] and IRF3 signaling [36]. The members of OTU superfamily vary in size between 230 and 1200 amino acid residues [28].

The members of OTU family select specific ubiquitin linkage types. Most of them cannot cleave linear ubiquitin. TRABID is a K63 specific DUB [12] whereas OTUB1 selectively cleaves K-48 linked polyubiquitin chains [37]. A20/TNFAIP3 cleaves K48 *in vitro* but its target protein is conjugated with K63-linked polyubiquitin chains [38, 39]. These family members are also associated with other ubiquitin-like protein as NEDD8 and ISG15 [37].

#### 1.3.1.2. Ubiquitin C-terminal Hydrolase (UCH) superfamily

The members of UCH family were the first deubiquitinating enzymes to be structurally identified [40, 41]. These family members are relatively small proteins, approximately 20-30 kDa compared to Ubiquitin Specific Proteases (USP). Their catalytic core domains which are located at the C-terminus are generally around 230

amino acids. They also have C-terminal extension with Cys, His and Asp residues. There are four members of this family: UCHL-1, UCHL-3, UCHL-5 and BRCA1 associated protein-1 (BAP1). UCHL-1 and UCHL-3 have functional roles in brain [42, 43] and it has been reported that UCHL-1 is associated with Parkinson's disease [44]. UCHL-5 plays a role in ubiquitin recycling [45]. Final member of this family, BAP1, is responsible for DNA repair [46].

#### 1.3.1.3. Machado-Josephin Disease proteases (MJDs) superfamily

This family consists of four members including ATXN3, ATXN3L, JOSD1 and JOSD2 [47, 48]. They contain highly conserved one cysteine and two histidine domains. In addition, JOSD1 and JOSD2 include josephin domain while ATXN3 and ATXN3L have additional ubiquitin interacting motifs (UIMs). ATXN3 is a well identified member of the MJD family. ATXN3 has two different Ub binding sites in the josephin domain at the N- terminus of the protein [49]. hence, ATXN3 cleaves both K48-linked and K63- linked polyubiquitin chains but prefers to cleave K63-linked polyubiquitin in the presence of UIMs [50]. It plays a role in polyubiquitin chain editing and so regulates folding and stability of target proteins [51]. It is also polyubiquitylated by itself [52].

#### 1.3.1.4. Ubiquitin Specific Proteases (USPs) Family

USPs are the largest group of DUB enzymes. Approximately 60 different genes in the human genome code for different deubiquitinating enzymes [28]. They contain around 350 amino acid catalytic core domains [53]. USPs have highly conserved regions called as Cys box (19 amino acids) and His box (60-90 amino acids). These boxes are available in catalytic domains of DUBs and they are important for deubiquitylation activity. They are large proteins due to their N- and C- terminal extensions besides insertions to catalytic domains. It was suggested that these insertion and extensions function to facilitate protein-protein interactions, target identification (Ubiquitin binding domains (UBDs) such as zinc finger ubiquitin

specific protease domain (Zn-UBP), ubiquitin interacting domains (UIMs) and ubiquitin associated domains (UBAs)) and subcellular localization of DUBs [54-56]. For instance, N-terminus extension of DOT4 (USP10) in yeast is responsible for binding to SIR4 protein [57]. Another study demonstrated that USP4 has a ubiquitin-like (UBL) domain at the N terminus of the catalytic domain. UBL domain, embedded in catalytic core region, is important for autoregulation of USP4 enzyme [58]. A transmembrane localization domain has also been identified in USP19 [59].

To date, 774 candidate proteins were identified as DUB interacting proteins [60]. These interactions can be divided into distinct classes. First interaction is between USPs and E3 ligases. This interaction regulates stability of E3 ligases by protecting from autoubiquitylation. For example, ICP0, a RING finger E3 ligase, interacts with USP7 enzyme and avoids autoubiquitylation *in vitro* and this interaction increases the stability of ICP0 E3 ligase [61]. Secondly, the USPs interact with a target of E3 ligase and these interaction directly regulates the stability of target protein E3 ligase. Some USPs interact proteins which regulate the activity of USPs such as UAF1 (USP1 associated factor 1) and GMPS (guanosine monophosphate synthetase) [62, 63]. Finally, USPs are also known to interact with each other. For example, USP7 and USP11 interaction stabilizes the Polycomb Repressive Complex 1 (PRC1) [64].

### 1.3.2. Metalloproteases DUBs

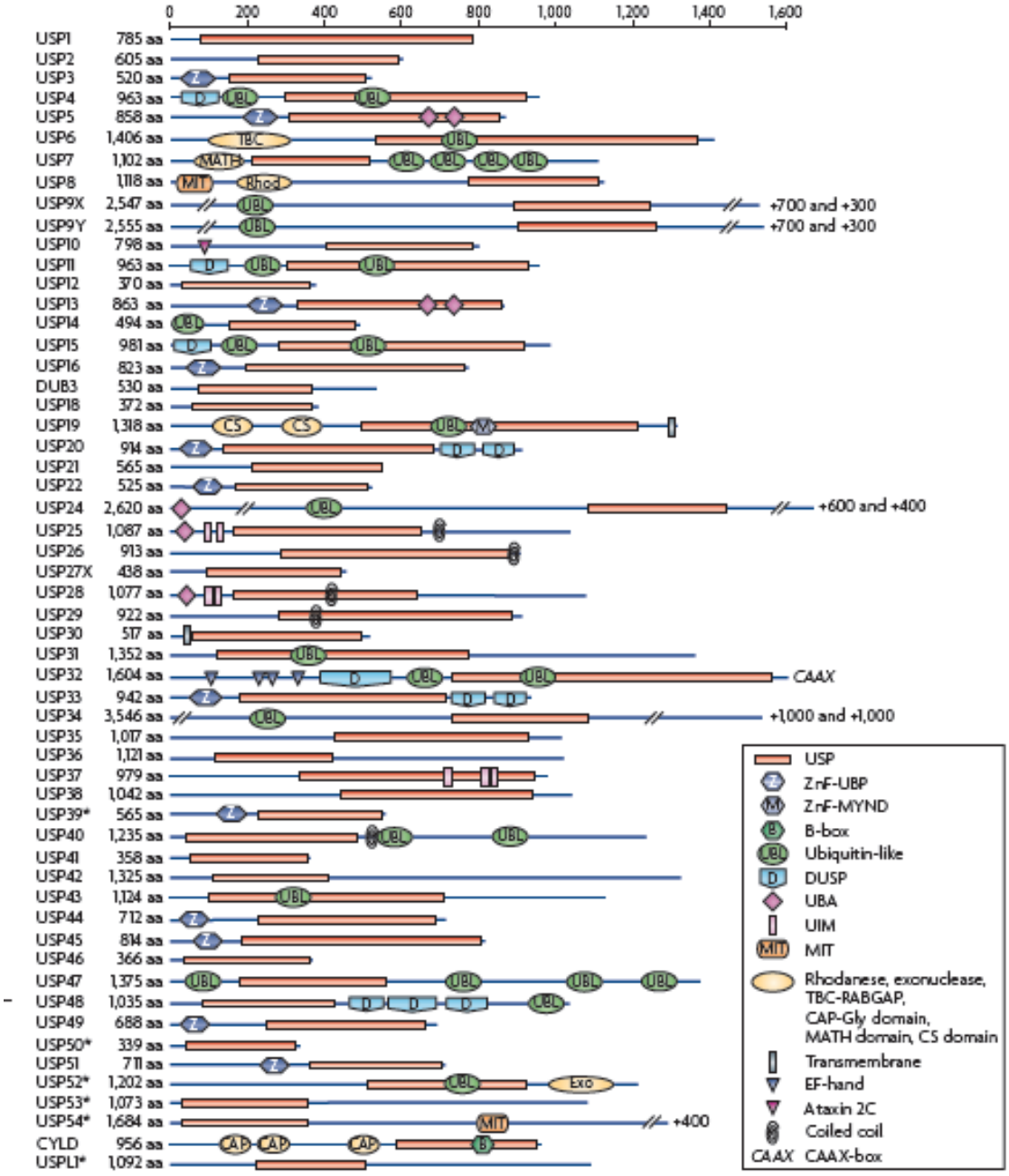
The active site of JAMM metalloprotease DUBs includes zinc binding site and zinc ions. These ions are connected with histidine, aspartate and serine residues. Zinc ions are responsible for activation of water molecules and lead to nuclephilic attack on isopeptide bond and target proteins are released. There is only one group in this family called as JAMM motif proteases (JAMMs).

#### 1.3.2.1. JAMM motif proteases (JAMMs)

There are eight different DUBs belonging to JAMMs family. One of them, PRPF8, is predicted as an inactive enzyme [65]. The rest of 7 members of this family are AMSH, AMSH-LP, BRCC36, POH1, CSN5, MPND and MYMS1. Structural analysis of AMSH and AMSH-LP showed that the DUBs in JAMM family cleave specifically K63-linked polyubiquitin chains [66]. Other studies experimentally verified JAMM family specifity for K63-linked polyubiquitin chains [67, 68].

JAMM family DUBs are generally active as part of large protein complexes. For instance, POH1 is associated with 26S proteosome and regulates recycling of ubiquitin chains [69]. In addition to POH1, AMSH and AMSH-LP, joining to ESCRT machinery, regulate membrane trafficking [70]. Furthermore, BRCC36 plays a role in DNA damage response as participating in BRCA1 complex [71, 72] and it is also associated with PA700 isopeptidase complex [73].

The domain structures of all DUBs are summarized in Figure 1.5.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USP1 785 aa
USP2 605 aa
USP3 520 aa
USP4 963 aa
USP5 858 aa
USP6 1,406 aa
USP7 1,102 aa
USP8 1,118 aa
USP9X 2,547 aa
USP9Y 2,555 aa
USP10 798 aa
USP11 963 aa
USP12 370 aa
USP13 863 aa
USP14 494 aa
USP15 981 aa
USP16 823 aa
DUB3 530 aa
USP18 372 aa
USP19 1,318 aa
USP20 914 aa
USP21 565 aa
USP22 525 aa
USP24 2,620 aa
USP25 1,087 aa
USP26 913 aa
USP27X 438 aa
USP28 1,077 aa
USP29 922 aa
USP30 517 aa
USP31 1,352 aa
USP32 1,604 aa
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USP36 1,121 aa
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USP46 366 aa
USP47 1,375 aa
USP48 1,035 aa
USP49 688 aa
USP50* 339 aa
USP51 711 aa
USP52* 1,202 aa
USP53* 1,073 aa
USP54* 1,684 aa
CYLD 956 aa
USPL1* 1,092 aa
+700 and +300
+700 and +300
+600 and +400
CAAX
+1,000 and +1,000
+400
MATH
MIT
Rhod
TBC
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CAP
USP
ZnF-UBP
ZnF-MYND
B-box
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DUSP
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CAAX-box

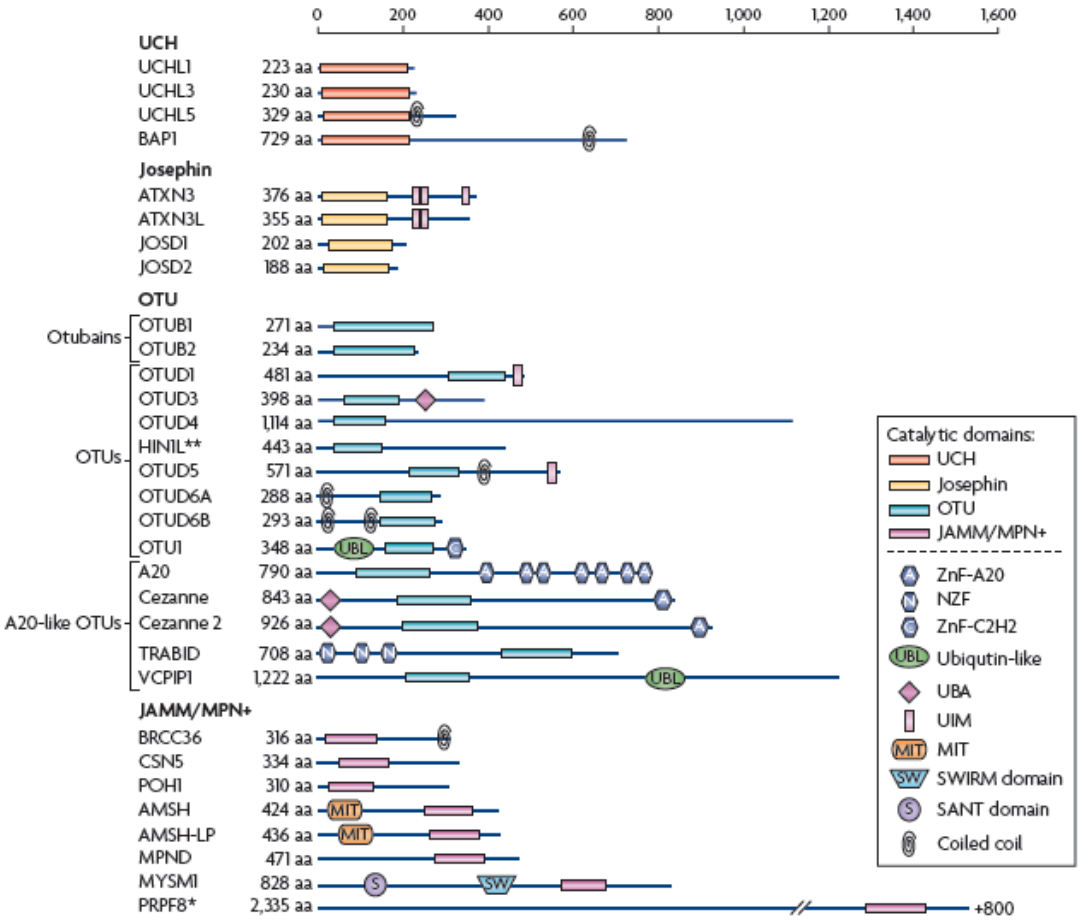



**Figure 1.5.** Schematic overview of domain structure of USP, UCH, OTU, MJD and JAMM families. DUBs include the zinc finger domains (ZnF-UBP, ZnFA20 or ZnF-C2H2) domain, the ubiquitin-interacting motif (UIM) and the ubiquitin-associated (UBA) domain. Ubiquitin-like (UBL) domains are also common in USPs. Furthermore, different DUBs contain additional domains according to their functions such as, microtubule-interacting and trafficking proteins (MIT), DUSP domain, EF-hand, Ca2+ binding motif, etc. * indicates the enzyme which are predicted as inactive. Figure is taken from [28]

## 1.4. Functions of DUBs

### 1.4.1. Catalytic function of DUBs

There are different catalytic functions of DUBs, including ubiquitin precursor processing, editing and recycling of ubiquitin. Known catalytic functions of DUBs are summarized in Figure 1.6.

#### 1.4.1.1. Processing of ubiquitin precursors

Ubiquitin is encoded from human genome as different types of fusion proteins [74]. These fusion proteins contain either several tandem repeats of ubiquitin [75] or fusion of ubiquitin and L40 or S27a ribosomal subunit [76, 77]. These polyubiquitins or ubiquitin fusion protein precursors cannot directly join to the free ubiquitin pool in cell. They must be processed by some highly active DUB enzymes (Figure 1.6a). In *Saccharomyces cerevisiae* many DUBs have roles in generating free ubiquitin from ubiquitin precursors as a free ubiquitin [78]

#### 1.4.1.2. Editing of Ubiquitin

Ubiquitin conjugates to ε-amino group of specific lysine residue of target proteins or to ubiquitin via its C-terminal glycine residue. This bond between ubiquitin and target protein or ubiquitin is called an isopeptide bond. DUBs which have been identified to date have isopeptidase activity and they can edit ubiquitin chains (Figure 1.6f). This activity of DUBs plays a role in the reversal of ubiquitylation or exchanging types of ubiquitin chains for another. p53 level in cell is mostly regulated by the editing ability of USP7 enzyme. p53 is ubiquitinated by MDM2 ubiquitin ligase. p53 is also regulated by the deubiquitinating enzyme. Expression of catalytically active and inactive form of USP7 can regulate stability of p53 [79-82]. Another example for ubiquitin editing function of DUBs is UCH37 (ubiquitin C-terminal hydrolase 37**)** enzyme. UCH37 cleaves Ub from the distal end of

polyubiquitin chains [83]. Therefore, UCH37 can prevent poorly ubiquitinated or slowly degraded Ub-linked protein from proteosomal degradation [84].

Ataxin3, belonging to MJDs family, also functions as a ubiquitin editing enzyme. Ataxin3 cleaves K63 and K48 mixed ubiquitin chains. Thus, Ataxin3 can facilitate proteosomal degradation of target protein and also interact with Ub-ligases to form specific linked ubiquitin chains [50].

Another example is A20, a Ub editing enzyme, which exchanges K63 linked polyubiquitin chains to K48 linked polyubiquitin chains of RIPK 1 (receptor-interacting serin threonine kinase I). this exchange results with degradation of RIPK 1 by proteosome [85].

#### 1.4.1.3. Recycling of ubiquitin

DUBs with isopetidase activity may also play a role in the recycling of ubiquitin. Ubiquitin recycling function of DUBs rescues ubiquitin from degradation together with target proteins in proteosomal and lysosomal pathways and maintain cellular ubiquitin homeostasis. Recycling function of DUBs has been firstly determined through the mutation analysis of yeast DUBs. UBP14 appears to have roles in the dissembly of unanchored ubiquitin and deletion of *UBP14* in yeast results in accumulation of unanchored ubiquitin, which competitively inhibits binding of degradation substrate to the proteosome [86].

Three other DUBs associated with 26S proteosome complex, UCH37, RPN11 and UBP6/UBP14 also contribute to recycling of ubiquitin. Target protein degradation must be conjugated to at least 4 ubiquitin to bind to the proteosome. UCH37 cleaves ubiquitin in order to shorten long ubiquitin chains to facilitate release of ubiquitin chain when proteosomal degradation of target protein is complete [87]. RPN11 is also part of the proteosome and functions as a recycling DUB. It can cleave the ubiquitin chain from isopeptide bond between substrate and ubiquitin [69, 88]. USP14 is the mammalian homolog of the yeast DUB enzyme Ubp6. Its catalytic

activity still remains unclear but it possibly functions in the shortening of ubiquitin chain [87, 89].

Taken together, DUBs can rescue target protein from degradation (Figure 1.6b) and remove non-degradative ubiquitin signal from target protein (Figure 1.6c). Hence, they also contribute to homeostasis of free ubiquitin pool in the cell (Figure 1.6e).

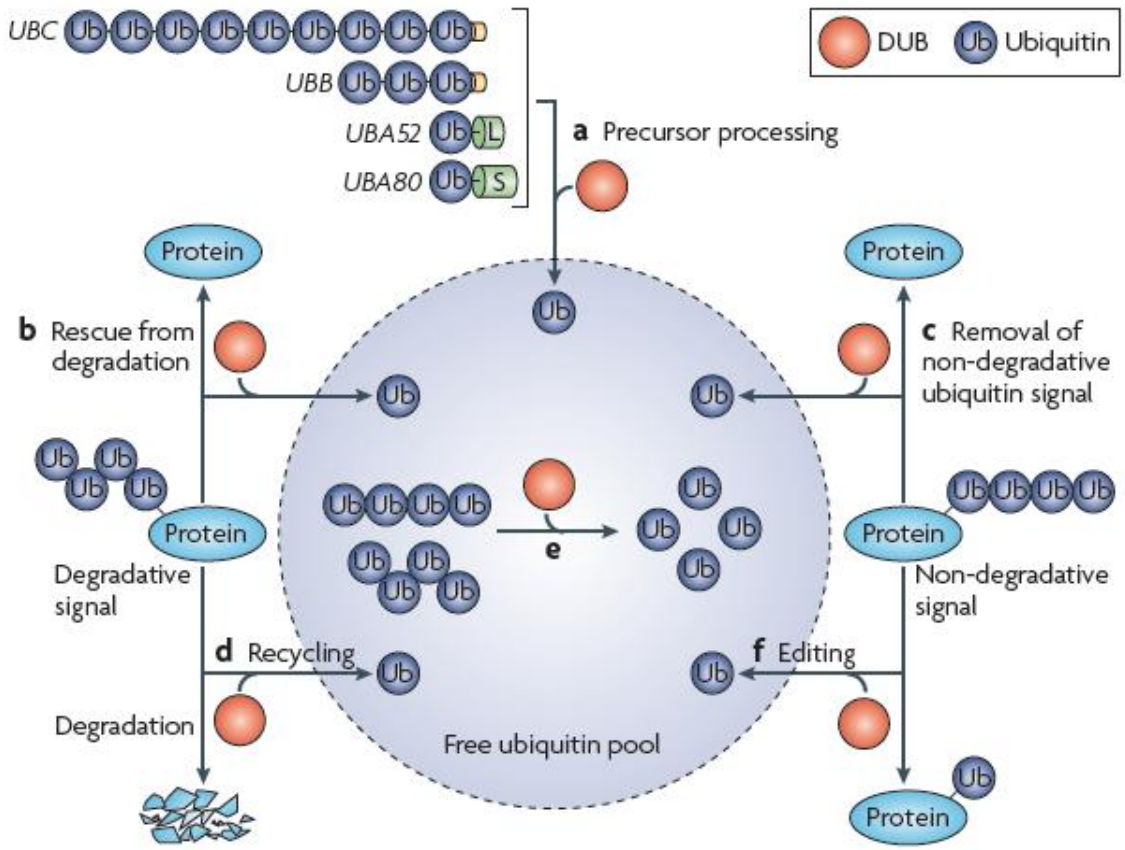



**Figure 1.6.** General catalytic function of DUBs. DUBs have crucial roles in processing of precursor fusion ubiquitin proteins to free ubiquitin **(a)**. DUBs can rescue target protein form degradation by removal of degradative signal **(b)** and they can remove non degradative signal **(c).** They have recycling function which refers to removal of ubiquitin signal from target protein when degradation process has been completed **(d).** DUBs also act as ubiquitin editing enzymes, and exchange ubiquitin signals **(f).** Finally, they contribute to free ubiquitin pool by disassembly of ubiquitin chains **(e).** Taken from [28].

### 1.4.2. Biological function of DUBs

DUBs have crucial roles in cellular events, including regulation of cell cycle, cell growth, development, DNA repair, chromatin remodeling, control of membrane trafficking, cell signaling and regulation of gene transcription.

DUBs have important roles in the regulation of cell cycle, cell growth and differentiation. USP8 (UBPY), for example, has roles in cell-cell contact induced-growth arrest and cell growth in fibroblasts [90]. UBP-M (USP16) is also known to be involved in cell cycle regulation. Expression of inactive form of UBP-M leads to cell cycle arrest and apoptosis possibly via deubiquitylation of histone H2A [91, 92]. USP28 stabilizes transcription factor MYC through FBW7 alpha protein which is part of the SCF type ubiquitin ligases [93]. Another example, USP39, is associated with spindle checkpoint function. Depletion of USP39 via RNAi results in decrease of Aurora B kinase. Therefore, this inhibits attachment of mitotic spindle to centromer [94]. USP7 is another cell cycle associated DUB. USP7 interacts with p53 and stabilizes it by deubiquitylation [79]. Decrease of USP7 expression level causes accumulation of p53 and cell cycle arrest [82, 95]. Recent study demonstrated that cell cycle progression is inhibited in *CSN5* deleted Mouse Embryo Fibroblasts (MEFs) at multiple steps because of enhancement of neddylation of cullin 1 and 4 and deregulation of expression many factors such as cyclin D and p53 by deletion of *CSN5* [96].

DUBs have also crucial roles in development. Fat facet (Faf) is a deubiquitinating enzyme in *Drosophila*, and is important for cell- cell interaction during eye development and oogenesis. [97, 98]. Fam is the homologous of Faf in mouse. Fam interacts with AF-6 during eye development in mouse. AF-6 is responsible for cell-cell adhesion during development through Ras protein binding ability [99, 100]. β-catenin which has role in cell junction and Wnt signaling pathway is also a substrate for Fam deubiquitylation enzyme and is stabilized by Fam [101-103]. The human homolog of Fam in mouse and Faf in *Drosophila,* a novel deubiquitylation enzyme, is USP9X. USP9X regulates different cellular events including cell growth,

proliferation and differentiation by interacting with mammalian target of rapamycine (mTOR) [104]. The homolog of yeast ubp14 in *Arabidopsis thaliana*, AtUBP14, has important roles in the disassembly of free polyubiquitin chains like UbpA. Deletion of AtUBP14 leads to death of embryos [105]. Finally, the members of ubiquitin carboxyl-terminal hydrolase subfamily, UCHL1 and UCHL3, are also important in mouse development. Wild-type UCHL1 and UHCL3 are related with oocyte cortex and meiotic spindle, respectively. They are associated with fertilization ability, sperm penetration and sperm incorporation [106].

Another newly emerging function of DUBs is in DNA repair. Several DUBs are involved in the regulation of DNA damage repair mechanisms. In Fanconi's anemia which is due to deficient DNA repair mechanisms, ubiquitylation and deubiquitylation of Fanconi anemia protein (FANCD2) has important roles in the control of DNA-repair mechanism. USP1 catalyzes deubiquitylation of monoubiquitylated FANCD2 and knockdown of USP1 leads to an increase of monoubiquitylated FANCD2 and prevents DNA damage [107]. USP1 is repressed by transcription factor p21 when the cell is exposed to a DNA damaging agent. Therefore, accumulation of monoubiquitylated FANCD2 helps to repair DNA damage [108].

The ubiquitylation and deubiquitylation of PCNA (Proliferating Cell Nuclear Antigen) are involved in DNA damage repair. Specific K164 residue of PCNA is monoubiquitylated or polyubiquitylated due to DNA damage. Ubiquitylation of PCNA can be reversed by USP1 deubiquitinating enzyme and depletion of USP1 causes the increase of mutagenesis level in the cell [109]. USP47 is an important deubiquitinating enzyme in BER (base excision repair) pathway. USP47 is involved in deubiquitylation of BER DNA polymerase β (Pol β) and provides stability of newly synthesized Pol β. New synthesized Pol β is immediately ubiquitylated and polyubiquitylated by E3 ligase Mule and CHIP in order to regulate its nuclear reserve. DNA damage induces inhibition of Mule and deubiquitylation of Pol β by USP47. Silencing of USP47 leads to the decrease of Pol β level through increase of

ubiquitylation of Pol β. Therefore, this cause accumulation of DNA strand breaks by blocking the BER pathway [110].

DUBs are known to have roles in chromatin remodeling. H2A and H2B, components of the nucleosome structure, are generally monoubiquitylated and monoubiquitylation of H2A is associated with gene silencing and X inactivation [111-113] whereas that of H2B are responsible for gene activation [114]. USP7 and USP11 are components of polycomb repressive complex (PRC1) which regulates silencing of gene expression by ubiquitylation of H2A histone. USP7 and USP11 control ubiquitylation level and turnover of MEL18 and BMI, both of which are PRC1 complex components and affect transcriptional regulation of $p16^{INK4a}$ [64]. MYSM1 (Myb-like, SWIRM and MPN domains 1) is a part of 2A-DUB complex. 2A-DUB complex also contains P/CAF which is a histone acetyltransferase and MYSM1 is associated with P/CAF. However, underlying mechanism of MYSM1 DUB activity by P/CAF remains unclear. A study showed that silencing of P/CAF leads to increased ubiquitylated H2A levels. H2A, which is associated with androgen receptor promoter genes, is deubiquitylated by MYSM1 and thus, MYSM1 promotes androgen receptor gene activation [115]. Similarly, USP22 also is a part of Spt-Ada-Gcn5-acetyltransferase (SAGA complex) and it regulates deubiquitylation and stability of telomeric-repeat binding factor 1 (TRF1) [116]. Like MYSM1, USP22 is also related with androgen receptor gene activation [117].

DUBs are also associated with membarane trafficking. Membrane trafficking is a crucial pathway to provide cellular organization and homeostasis. Endosomal sorting complex required for transport (ESCRT) machinery is important for transport of ubiquitylated receptors from endosome to lysosome through multivesicular bodies (MVBs) [118, 119]. The best characterized two DUBs associated with ESCRT are AMSH (STAMBP, STAM binding protein) and USP8. AMSH regulates epidermal growth factor receptor (EGFR). Depletion of AMSH using RNAi increases the degradation of EGFR [120].[121]. AMSH also play a role in regulation of the chemokine receptor CXCR4 stability and trafficking. Inactive AMSH or depletion of AMSH by RNAi causes increase of level of CXCR4

receptor because CXCR4 receptor stability and degradation are regulated by ESCRT-0 Hrs and STAM1 components and catalytically inactive form of AMSH is associated with hyperubiquitylation of ESCRT-0 component STAM1 and Hrs [122]. The effect of USP8 on EGFR trafficking have reported controversial results. Some studies showed that depletion of USP8 inhibits degradation of EGFR and EGFR deubiquitination of EGFR by USP8 is important for EGFR degradation [123, 124]. Some other studies examined that USP8 negatively regulates EGFR degradation by deubiquitylation [125].

Many DUBs regulate different signaling pathways which play role in pathogenesis of severe diseases, including cancer and neurodegenerative diseases. The most important signaling pathways associated with DUBs are TGF-β, NF-κB and Wnt-signaling pathways.

TGF-β (Transforming Growth Factor-Beta) signaling is associated with cell proliferation, apoptosis, embryonic development and tissue homeostasis [126]. Silencing of USP9X leads to activity of E3 ligase ectodermin and subsequently accumulation of monoubiquitylated SMAD4 in TGF-β pathway [127]. USP15 deubiquitylates monoubiquitylated regulatory-SMADs (R-SMADs) and remove ubiquitin from DNA binding domains of R-SMADs. Therefore, USP15 has a crucial role in the regulation of target promoters by R-SMADs [128].

DUBs are also associated with Wnt signaling which is important pathway in embryonic development and cancer progression, which controls cell-cell interaction [129]. USP15 interacts with COP9 signalosome (CSN) which regulates Wnt/β-catenin signaling pathway and balance APC and β-catenin [130]. USP4 is associated with Nemo-like kinase leading to nuclear accumulation of USP4. Accumulation of USP4 results in deubiquitylation of T-cell factor 4 (TCF4) transcription factor. Thus, USP4 blocks β-catenin-dependent transcription [131].

Another important pathway associated with DUBs is NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) signaling pathway which plays a role in

innate and adaptive immune responses. A20 regulates deubiquitylation of TRAF6 [132] and proteosomal degradation of RIP1 through editing of ubiquitin chains from K63 to K48 polyubiquitylation [133]. USP2 is important for phosphorylation of IKBα and is a positive regulator for NF-κB signaling [134] where as USP11 negatively regulates NF-κB by targeting IKBα [135, 136].

Known biological functions of DUBs are summarized in Figure 1.7.

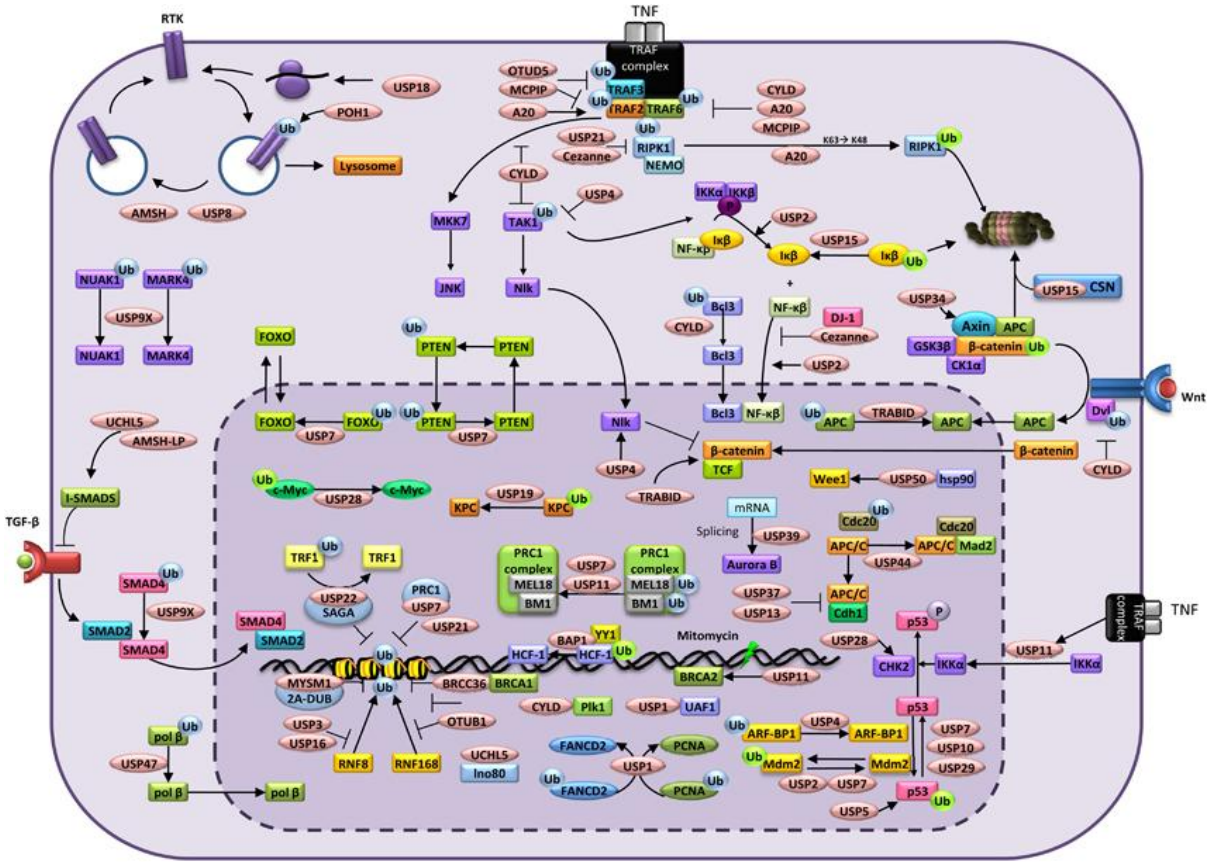



**Figure 1.7.** Summary of biological functions of DUBs. DUBs have crucial roles in important cellular events including cell cycle regulation, differentiation, development, DNA repair, signal transduction, etc. Taken from [137].

## 1.5. Ubiquitin Specific Proteases 32 (USP32)

*USP32* (Accession number: NT_010783 and mRNA accession number: NM_032582) is located on chromosomal band 17q23 which is a frequent site of gene amplification in breast cancer [138, 139]. Several genes have been identified in this chromosomal region as oncogenes and these genes contribute to tumor development. First oncogene identified in this region is *RPS6KB1* (ribosomal protein S6 kinase, 70kDa, polypeptide 1) which is amplified in 8-10% primary breast tumors [140]. *TBX2* (T-box transcription factor) and *PPM1D* (protein phosphatase, Mg2+/Mn2+ dependent, 1D) are oncogenes which are located in 17q23 amplicon and are highly amplified and overexpressed in breast cancer cell lines [141, 142]. 17q23 chromosomal band also harbors several candidate oncogenes including *APPBP2* (amyloid beta precursor protein (cytoplasmic tail) binding protein 2), *BRIP1* (BRCA1 interacting protein C-terminal helicase 1), *POPX1* (protein phosphatase, Mg2+/Mn2+ dependent, 1E) and *USP32* (ubiquitin specific protease 32).[142-144]. Therefore, amplification and overexpression of oncogene candidate genes in this region could be important for breast cancer.

During evolution, *USP32* gene was highly conserved and its fusion with *TBC1D3* (TBC1 domain family, member 3) leads to the formation of USP6 which is a proto-oncogene deubiquitinating enzyme [145]. *USP6* mRNA has 89% (1-3193 nucleotides) and 97% (3194-6063 nucleotides) sequence similarity to *TBC1D3 and USP32*, respectively [145].

*USP32* encodes a 1604 aa novel predicted deubiquitinating enzyme (Protein accession number: NP_115971.2). USP32 harbors five conserved domains including, $Ca^{+2}$ binding domain with EF-hand motif between 189-339 and 237-293 amino acids (aa), DUSP domain (domain present in ubiquitin-specific proteases) between 548 and 712 aa. The 3 peptidase domains are between 733-911, 1225-1313 and 1510-1565 aa positions. Cys and His domains which are common in USP subfamily of DUBs are found between 733-755 and 1510-1543 (Figure 1.7).

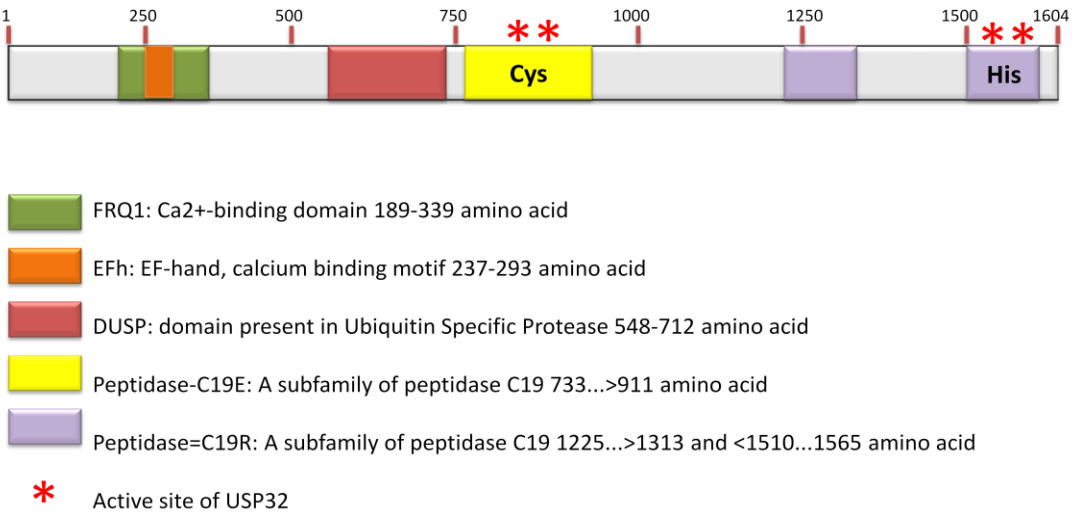



**Figure 1.8.** Schematic overview of predicted USP32 domain structure.

DUBs are known to have roles in cancer pathogenesis and are considered as potential drug targets. For example, overexpression of USP22 is associated with colorectal carcinoma and can be used as a marker gene for human cancer [146]. USP10 levels are associated with tumor aggressiveness and invasion properties of cancer cells [147].

Taken together, USP32 is an uncharacterized deubiquitinating enzyme and located in an amplicon region which may be important for breast cancer development. USP32 also has sequence similarty to USP6 which is an oncogene. Functional and biochemical characterization of USP32, an oncogene candidate, may help us understand its potential role in normal and cancer cells

## 1.6. Aim of the study

In this study, we focused on functional and biochemical analysis of an uncharacterized, predicted DUB, USP32. Bioinformatics tools, NCBI Conserved Domain Tools, ExPASy and, Simple Modular Architecture Research Tool (SMART), suggested that USP32 has C-terminal Cys and His box domains that are common in deubiquitinating enzymes. In addition, a comparison of amino acid sequences of known DUBs (USP6, Doa4) revealed conserved residues that match with the Cys-His domains of USP32. Given that DUB, Cys and His domains are found in active DUBs, we aimed to investigate if USP32 is indeed an active DUB which is as of yet uncharacterized. To test the DUB activity, we performed *in vivo* and *in vitro* assays and further investigated its biochemical properties such as catalytic activity and kinetic properties.

# CHAPTER II

# MATERIAL AND METHODS

## 2.1. Agarose gel electrophoresis and gel extraction

Agarose (1%, w/v) in 1X Tris-Borate-EDTA (TBE, Appendix C) or 0.5 X Tris-Acetate-EDTA (TAE, Appendix C) buffer was boiled until completely dissolved. Ethidium Bromide at final concentration of 0.5 µg/ml was added to agarose. 10X loading dye was diluted with DNA sample at a final concentration of 1X. The suitable DNA marker (Appendix D) was used to determine the size of DNA. Agarose gel electrophoresis was performed at 100 volts. Finally, DNA bands on agarose gel were visualized and documented under UV light. Size-confirmed DNA fragments were cut from the agarose gel and DNA bands were extracted using Agarose gel extraction kit (Roche) according to manufacturers' instructions.

## 2.2. Preparation of competent cell

DH5α or Top 10 *E. coli* cells were grown in 10 ml LB media in a 50 ml falcon tube at 37°C for overnight to prepare a starter culture. From the starter culture, 300 µl was used to inoculate a 50 ml LB media. Cultures were grown at 37°C for 2-3 hours, until $OD_{600}$ reached to 0.6. Bacterial culture was then separated into 2 sterile pre-chilled centrifuge tubes. The tubes were incubated on ice for 10 minutes and then centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded from the tubes. The pellet was resuspended in 5 ml of 10 mM fresh and pre-chilled $CaCl_2$ by vortexing. The bacterial pellet treated with $CaCl_2$ was centrifuged for 10 minutes at 3000 rpm at 4°C. The supernatant was discarded again and the pellet was resuspended in 1 ml of 75 mM fresh and pre-chilled $CaCl_2$. Then, 200 µl of ice-cold

100% glycerol was added. The competent bacterial cell solution was divided into 1.5 ml eppendorf tubes and quickly frozen using liquid nitrogen. The aliquots were stored at -80°C.

### 2.3. Transformation

The chemically competent *E. coli* cells were thawed on ice. 4 µl of each ligation reaction and 10-100 ng of control plasmids were added to 50-100 µl competent cells and incubated on ice for 30 min. The cells were then incubated at 42°C for 45 seconds and immediately placed on ice. After 2 min, 450 µl S.O.C (Super Optimal Broth with Catabolic repressor, Appendix C) or LB (Luria-Bertani, Appendix C) media were added to each reaction tube. The cells were incubated at 37°C and shaked at 200 rpm for 1 h. 250 µl of the transformed cells were spread on LB-Agar plates (Appendix C) containing appropriate antibiotics and the plates were incubated at 37°C for 16 h.

### 2.4. Preparation of glycerol stock

The bacterial cells were mixed with autoclaved glycerol (15% of total volume) in 1.5 ml tubes. The mixture was gently mixed by inverting and was frozen in liquid nitrogen and immediately transferred to -80°C freezer. For future uses, the culture was scraped on its surface using a sterile loop and was inoculated into a new LB media with appropriate antibiotics. The glycerol stock was put back immediately at -80°C to avoid thawing.

## 2.5. Investigation of *in vivo* deubiquitinating enzyme activity of USP32

### 2.5.1. Subcloning of *USP32*

#### 2.5.1.1. Primer design for amplification of partial fragments of USP32 catalytic domain

The *USP32* gene sequence and USP32 conserved domain information were obtained from NCBI (National Center of Biotechnology Information). Specific primers (Appendix A) with *Sal I* and *Not I* recognition and restriction sites were designed and used for amplification of partial fragments of USP32 into pGEX-4T-2 vector (Appendix B). Additional nucleotides were added to forward primers following the restriction enzyme recognition site to maintain the open reading frame of the GST ( Gluthation-S-transferase) gene sequence in the pGEX-4T-2 vector.

All primers designed for the amplification of partial fragments of USP32 catalytic domains are summarized in Table 2.1.

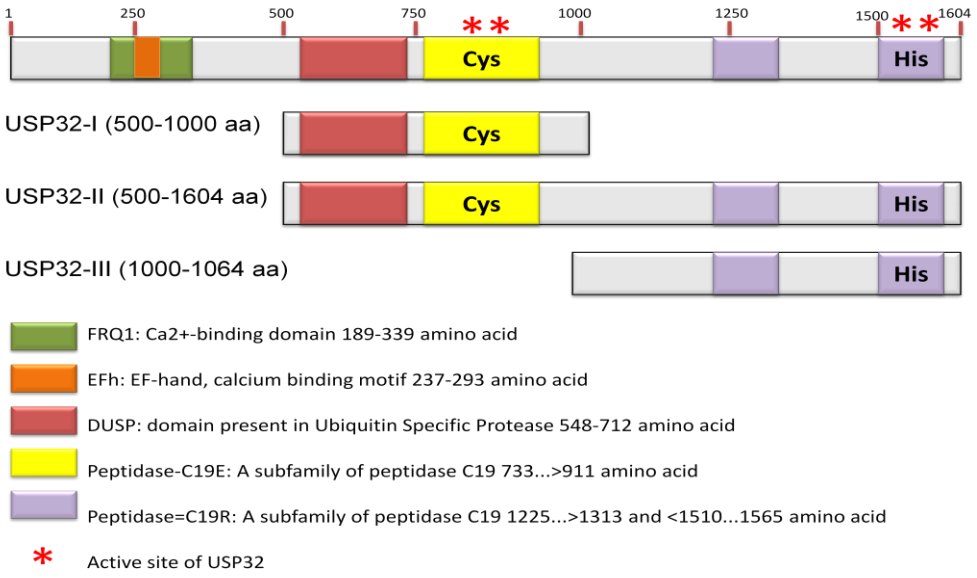



**Figure 2.1.** Schematic outlines of conserved sequence motifs and 3 cloning fragments of USP32. ** shows the active sites named as Cys and His domains. USP32 protein has 1604 amino acids.

**Table2.1.**Cloning primer sequences for the USP32 catalytic domain and full length constructs (5'→3')

| Primer Name | Recognition site | Restriction site | Extra nucleotide | USP32 sequence |
|---|---|---|---|---|
| USP32 F1 | ACGC | GTCGAC | T | AATAACAACCAGTGTTTGCT |
| USP32 R1 | ATAAGAAT | GCGGCCGC | - | TTAGAGGCTGGGGCGATTCTT |
| USP32 F2 | ACGC | GTCGAC | T | GTAACTCAAGAACCAGTAAA |
| USP32 R2 | ATAAGAAT | GCGGCCGC | - | TTACTGTAACACACAGTACTTT |
| USP32 F3 | ACGC | GTCGAC | T | CTGGGTGTCTCCAATTTCAGCT |
| USP32 R3 | ATAAGAAT | GCGGCCGC | - | TTACTGTAACACACAGTACTTT |

* Nucleotides in red indicate the stop codons.

### 2.5.1.2. PCR Amplification of USP32 fragments

Two partial fragments of USP32 either with the Cys domain (USP32-I (500-1100 aa)) or His domain (USP32-II (500-1604 aa)) along with a construct with both of the domains ((USP32-III (1000-1604 aa)) were generated using the above mentioned primers (Table 1). PCR reaction mixture was prepared with 1X reaction buffer, 100 µM of each dNTP, 400 nM forward and reverse primers and 2.5U of Expand High Fidelity$^{plus}$ PCR System (Roche) was added to each reaction. Reaction volume was completed to 50 µl using sterile $dH_2O$. PCR conditions were optimized for each fragment as shown in Table 2.2.

**Table 2.2.** PCR condition for USP32 constructs (I, II and III)

| | USP32-I and USP32-III | USP32-II |
|---|---|---|
| Initial denaturation | 94$^{o}$C for 2 min | 94$^{o}$C for 2 min |
| Denaturation | 94$^{o}$C for 30 min | 94$^{o}$C for 30 min |
| Annealing | 72$^{o}$C for 30 min | 72$^{o}$C for 30 min |
| Extension | 72$^{o}$C for 2 min | 72$^{o}$C for 4 min |
| Final Extension | 72$^{o}$C for 7 min | 72$^{o}$C for 7 min |

### 2.5.1.3. Cloning of USP32-I and USP32-II into pGEX-4T-2

USP32-I (500-1100 aa) and USP32-II (500-1604 aa) were cloned into pGEX-4T-2 vector using *Sal I* and *Not I* restriction enzymes (Fermentas). Conserved domain of full length USP32 and cloned partial constructs are shown in Figure 2.1. PCR products of *USP32* fragments purified from agarose gel and pGEX-4T-2 vector were digested with *Sal I* and *Not I* restriction enzymes at 37 $^{o}$C for overnight incubation. Digestion products were run on 0.8% agarose gel and extracted from gel using agarose gel extraction kit (Roche). In addition, digested pGEX-4T-2 vector was treated with Alkaline Phosphatase (Roche) and purified using phenol:chloroform:isoamylalcohol (25:24:1) extraction and ethanol precipitation methods. Purified vector (100 ng) and *USP32* fragments were ligated at 16 $^{o}$C for 16

h using T4 DNA ligase (Roche). A few microlitres of the ligation reaction was directly transformed into Top10 or DH5α cell.

USP32-II fragment (Figure 2.1) was amplified as described above. 3'A overhangs was added to the blunt-end PCR product and incubated at 72 $^{o}$C for 15 min with Taq DNA polymerase enzyme (Fermentas) and 2 mM dATP after PCR reaction. TOPO-TA cloning was performed with 2 µl PCR product with 3'A overhangs, 1 µl salt solution (1.2 M NaCl and 0.06 M $MgCl_2$), 1µl TOPO-TA vector (PCR 8/GW/TOPO) (Appendix B) and 2 µl sterile water. Reaction was mixed gently and incubated for 15 min at room temperature. 2 µl of TOPO-TA cloning reaction was used for transformation into Top 10 *E.coli* strain. The colonies obtained from the transformation were used for plasmid isolation using high pure plasmid isolation kit (Roche). The USP32-II construct was confirmed using restriction digestion with Sal I and Not I restriction enzyme (Fermentas).

The sequences of USP32-I, II, III fragments were sequence verified using vector specific primers (Appendix A) (Iontek, Istanbul).

#### 2.5.1.4. *In vivo* Deubiquitination assay in *E.coli*

The deubiquitination assay is based on the cleavage of ubiquitin-β-galactosidase (ub-β-gal) fusion proteins by an active deubiquitinating enzyme [78, 148]. Ub-met-β-gal was expressed from a pACYC184-based plasmid (a gift from Dr. M. Hochstrasser). PACYC184-Ub-met-β-gal construct and pGEX-4T-2-USP32-I, pGEX-4T-2-USP32-II and pGEX-4T-2-USP32-III containing USP32 fragments and pGEX-UBP3 constructs were co-transformed into DH5α *Escherichia coli*. pGEX-UBP3 (a gift from Dr. M.Hochstrasser) was used as positive control for deubiquitinating enzyme activity [78]. Colonies that contain PACYC184-Ub-met- β-gal and GST fusion construct were selected using LB media with 100 µg/ml final concentration of ampicillin and 10 µg/ml final concentration of chloramphenicol. Co-transformed *E.coli* DH5α cells were grown in 2X YTA (2X Yeast extract and Tryptone+100 µg/ml ampicillin) , Appendix C) media until $OD_{600}$ reached to 0.6-0.8.

Isopropyl-1-thio-ß-Dgalactopyranoside (IPTG) was then added to a final concentration of 0.2 mM because the expressions of USP32-I, II, III fragments in the pGEX-4T-2 vector were inducible with IPTG. After 4 h of incubation with IPTG at 30 $^{o}$C, cells were lysed in 1X PBS buffer containing 100 µg/ml Lysozyme, protease inhibitor (Roche Complete Mini Protease Inhibitor Cocktail Tablets -1 tablet in 10 ml 1XPBS buffer) and 10 U/ml DNase I using the freeze/thaw method [149] and total proteins were separated by 10% SDS-PAGE. Protein lysates were analyzed by immunoblotting with monoclonal mouse anti-β-galactosidase antibody with 1:1000 dilution (Cell Signaling), goat anti-mouse IgG-HRP secondary antibody with 1:2000 dilutions (Santa Cruz) and ECL system (Pierce). The same samples were verified by immunoblotting with goat anti-GST antibody with 1:2000 (Amersham) and rabbit anti-goat IgG-HRP secondary antibody with 1:10000 (Santa Cruz).

## 2.6. Investigation of *in vitro* deubiquitinating enzyme activity of USP32

### 2.6.1. Primer design for Ligation independent (LIC) cloning of USP32 full length (USP32-FL) and catalytic domain (USP32-CD)

Primers were designed using Protein Crystallization Construct Designer program from Netherlands Cancer Institute (NKI) (Protein CCD at NKI-http://xtal.nki.nl/ccd/start_ccd.html ) [150]. To generate compatible overhangs for pFastNKI-his3C-LIC vector, CAGGGACCCGGT and CGAGGAGAAGCCCGGTTA sequences was added to forward and reverse primer at their 5'end, respectively. Primer sequences USP32-FL and USP32-CD are shown in Appendix A.

### 2.6.2. Cloning of USP32-FL and USP32-CD into pFastNKI-his3C-LIC vector via LIC cloning strategy

USP32-FL (Full length) and USP32-CD (catalytic domain) constructs were cloned into pFastNKI-his3C-LIC vector (Appendix B) for expression in insect cells as

previously described [151]. After creating vector-compatible 5' sequence of USP32-FL and USP32 CD were amplified with PCR, USP32-FL and USP32 CD were cloned into pFastNKI-his3C-LIC using LIC cloning strategy as displayed at Figure 2.2. vector and insert preparation for LIC cloning are given in Table 2.3. After preparation of vector and insert, 2 µl of insert (40 ng) and 1 µl of vector (10-20 ng) were mixed and incubated at room temperature for 5 min. 3 µl of mixture was transformed into DH5α cells. Transformed bacteria were spread on LB agar media with 100 mg/ml ampicillin antibiotic.

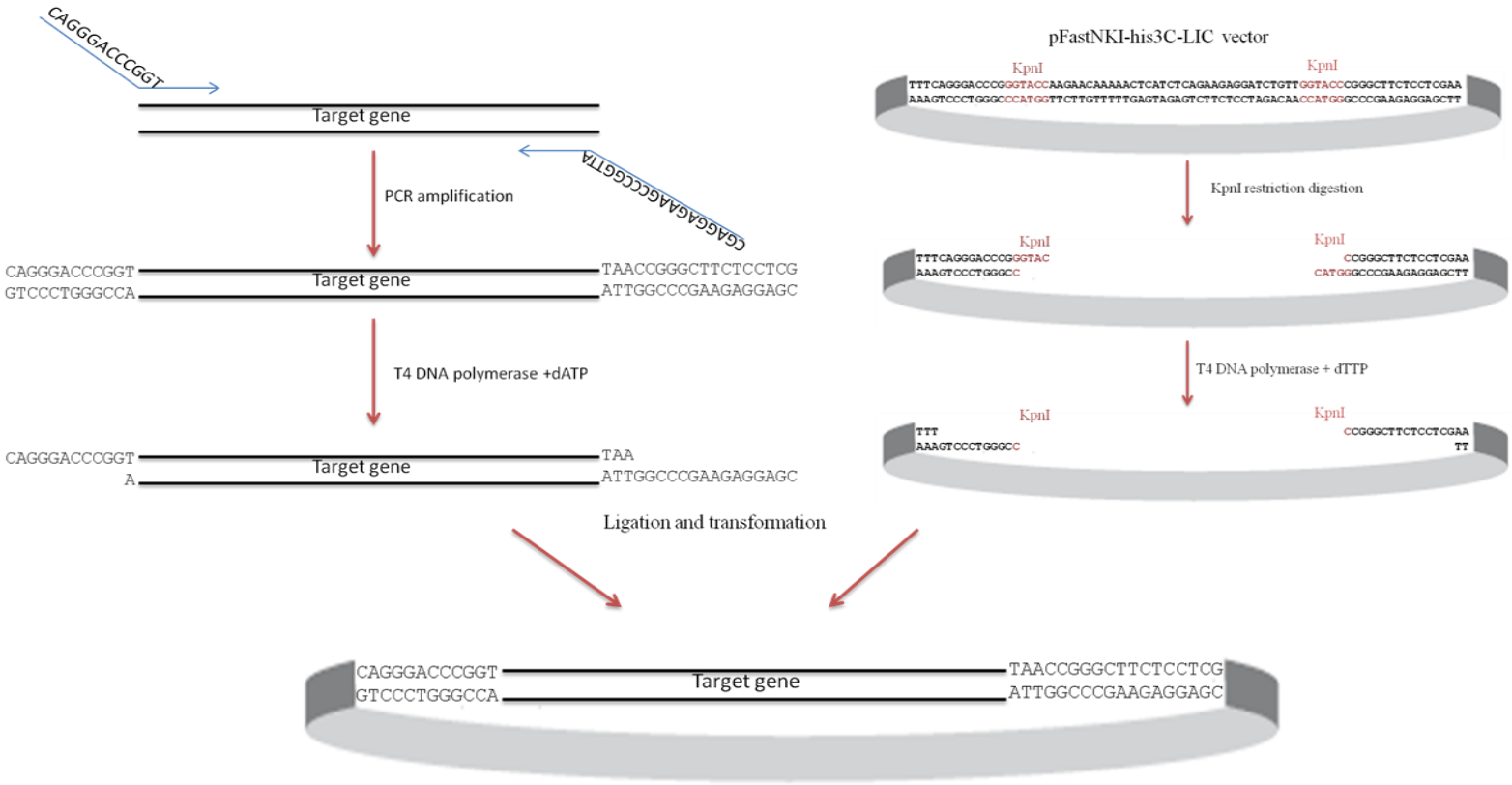



**Figure 2.2.** Ligation independent cloning (LIC) strategy.

**Table 2.3.** Vector and Insert preparation reactions for LIC cloning

| Vector preparation | Insert Preparation |
|---|---|
| ~400 ng KpnI digested vector | 100-200 ng PCR product (purified from gel) |
| 1µl NEB1 buffer | 2µl NEB1 buffer |
| 1µl 25mM dTTP | 2µl 25mM dATP |
| 1µl T4 DNA polymerase | 2µl T4 DNA polymerase |
| Complete the volume to 10 µl with $H_2O$ | Complete the volume to 20 µl with $H_2O$ |
| Reactions were incubated at room temperature for 30 min and inactivated at 75 $^oC$ for 20 min. | |

### 2.6.3. Confirmation of mutation-free pFastNKI-his3C-LIC-USP32-FL and pFastNKI-his3C-LIC-USP32-CD constructs

The colonies were selected from transformation plates and were grown in LB media with 100 mg/ml final concentration of ampicillin at 37 $^oC$ overnight. Glycerol stocks were prepared from overnight cultures. Cells were harvested to isolate the plasmids using high pure plasmid isolation kit (Roche). pFastNKI-his3C-LIC-USP32-FL and pFastNKI-his3C-LIC-USP32-CD constructs were verified with NcoI restriction enzyme (Roche) digestion. The constructs verified via restriction digestion were used for sequencing using primers vector and *USP32* specific primers (Appendix A) and at the SequenceCore facility at NKI.

### 2.6.4. Protein expression in insect cells

#### 2.6.4.1. Insect cell culture

*Spodoptera frugiperda* Sf9 or Sf21 insect cells were used as hosts for the baculovirus. SF900 II SFM media (Invitrogen) containing 1% Penicillin-Streptomycin was used for insect cell culturing. Suspension insect cells were grown under serum-free conditions at 27$^oC$ with shaking.

### 2.6.4.2. Bacmid preparation

200 ng of purified and verified pFastNKI-his3C-LIC-USP32-FL and pFastNKI-his3C-LIC-USP32-CD plasmids were added to 100 µl of MAX efficiency DH10Bac chemically competent cells. The cells were incubated at room temperature for 30 min and then heat-shocked at 42 $^{o}$C for 45 sec. Reaction tubes were immediately transferred to ice and incubated for 2 min. 900 µl of S.O.C media at room temperature was added into tubes and the cells were grown at 37 $^{o}$C for 4 hours at 225 rpm. After 4 h, 10 times serial dilutions ($10^{-1}$, $10^{-2}$, and $10^{-3}$) were prepared from transformed cells using S.O.C media (Appendix B). 100 µl of each dilution was spread on LB agar plate containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, 10 µg/ml tetracycline, 100 µg/ml Bluo-gal, and 40 µg/ml IPTG and incubated at 37 $^{o}$C for 48 h to screen blue/white colonies. After 48 h, 10 different white colonies were selected and restreaked on LB agar and incubated at 37 $^{o}$C for overnight. Reconfirmed white colonies were grown at LB media containing 50 µg/ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline and were then used for bacmid isolation using HighPure plasmid isolation kit (Qiagene). Bacmids were divided into aliquots and stored at -20 $^{o}$C.

### 2.6.4.3. Bacmid transfection into insect cells

Sf9 or Sf21 insect cells were transfected with 10 µg bacmid using Cellfectin II reagent (Invitrogen). 1x$10^{6}$ cells/ml insect cells in 3 ml culture media were seeded into 6-well plates and incubated at 27 $^{o}$C for 30 min for cell attachment. Meanwhile, 8µl Cellfectin II reagent was diluted with 100 ml SF900 II media without serum and antibiotics and also 10 µg bacmids were diluted with 100 ml SF900 II media without serum and antibiotics. Dilutions of bacmids and transfection reagents were combined and incubated at room temperature for 30 min after gently mixed. DNA-Cellfectin II mixture was added on cells by dropping. After 3-5 hours incubation at 27 $^{o}$C, transfection media was replaced with complete media containing antibiotics. After 3 days, P1 baculovirus stock was collected from culture media. 1x$10^{6}$ cells/ml

insect cells in 30 ml media was infected with P1 baculovirus stock to prepare P2 baculovirus stock. Baculovirus stocks were stored at 4$^{o}$C and protected from light.

#### 2.6.4.4. Protein expression and purification

1x10$^{6}$ Sf9 or Sf21 Sf9 or Sf21 insect cells were infected using a low-MOI (Multiplicity of Infection-ratio of infectious virus particles) to cells infection method [152]. The cells were harvested 72 hours after the baculovirus infection. All steps for generation of baculovirus and expression of protein of interest are explained in Figure 2.3. Cells were lysed with lysis buffer (20 mM Tris pH: 8.0, 500mM NaCl, 5mM B-mercaptoethanol, 10 mM imidazole and protease inhibitor cocktail) and sonication. The lysates were centrifuged at 20000 rpm for 30 minutes at 4C. The supernatants were incubated with washed Talon metal affinity resin (Clontech, Inc., Palo Alto, CA) for 20 minutes at 4$^{o}$C and the beads were then washed with wash buffer (20mM Tris pH: 8.0, 500mM NaCl, 5mM B-mercaptoethanol and 10 mM imidazole). Protein was then eluted with elution buffer containing 20 mM Tris pH 8.0, 500 mM NaCl, 5 mM B-mercaptoethanol and 250 mM imidazole. Proteins were dialyzed to remove imidazol and purified with size exclusion column (S200 16/60 column) using AktaPrime. All proteins were stored at -80$^{o}$C.

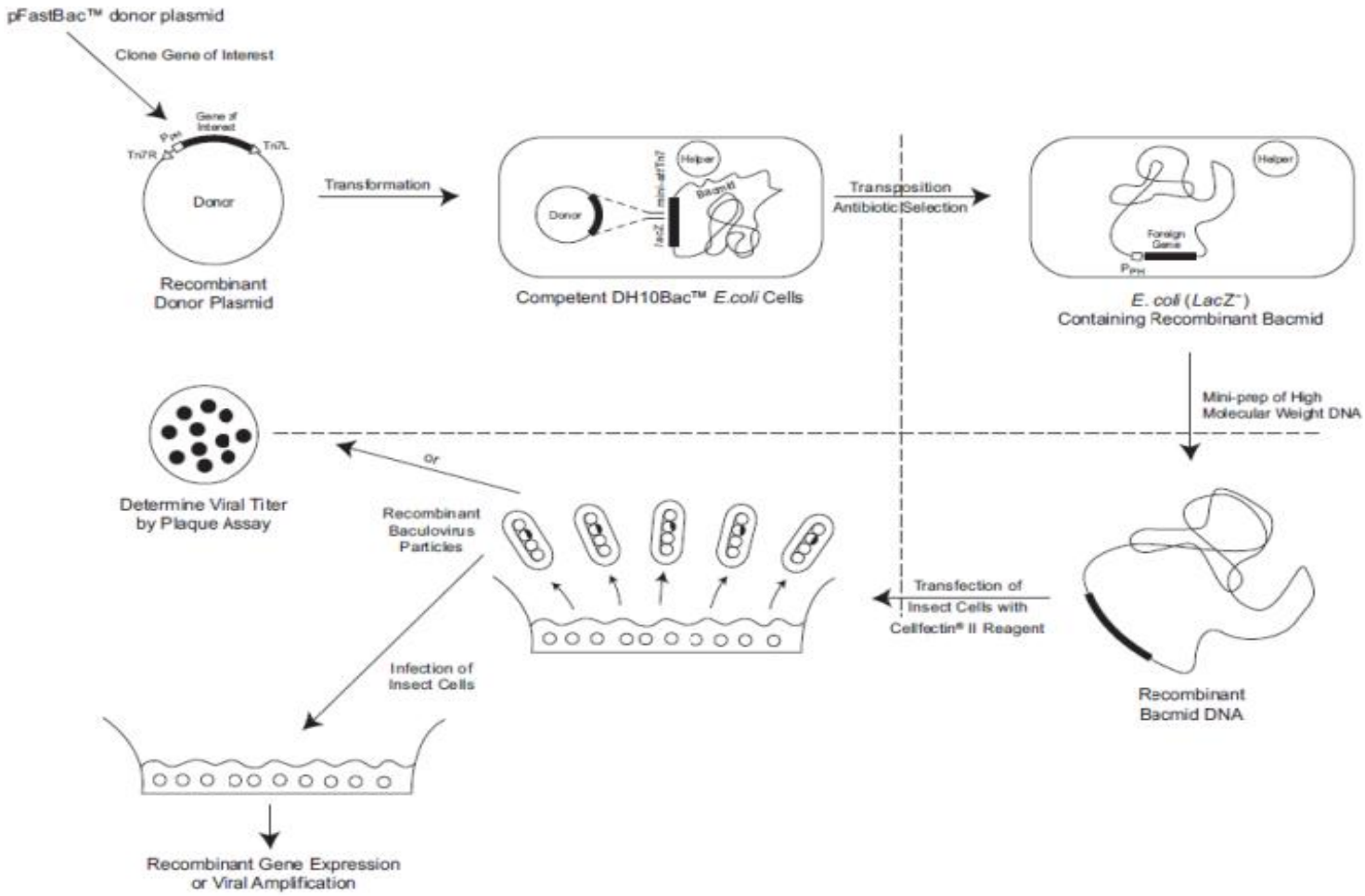



**Figure 2.3.** Generation of baculovirus stocks and expression of the protein of interest in insect cells. (taken from the Invitrogen Bac-to-Bac Baculovirus Expression System User Manual).

### 2.6.5. *In vitro* deubiquitination assay

*In vitro* deubiquitinating enzyme activity was performed using gel-based TMR-Ub-VME probe labeling and fluorescent-based Ub-Rho assay.

#### 2.6.5.1. Gel based-TMR-Ub-VME (Tamra-Ubiquitin-Vinyl methyl ester) labeling

Different concentrations of USP32-FL and USP32-CD (100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.1 nM, 1.6 nM, and 0.8 nM) were used for TMR-Ub-VME labelling. 250 μM final concentration of NMM DUB inhibitor was added to the 100 nM enzyme containing reaction. DMSO was added to other tubes instead of the NMM inhibitor. Mixtures were incubated at 37 $^{o}$C for 30 min. 0.5 mg/ml final concentration of TMR-Ub-VME probe was added to tubes after incubation with NMM inhibitor and all reactions were incubated at 37 $^{o}$C for 30 min. Reaction was stopped by adding NuPAGE LDS sample buffer (Invitrogen). Samples were loaded and run on 4-12 % Bis-Tris NuPage gel (Invitrogen). Gel was visualized on PerkinElmer ProExpress 2D Proteomic Image System using 535 nM excitation and 595 nM emission filters.

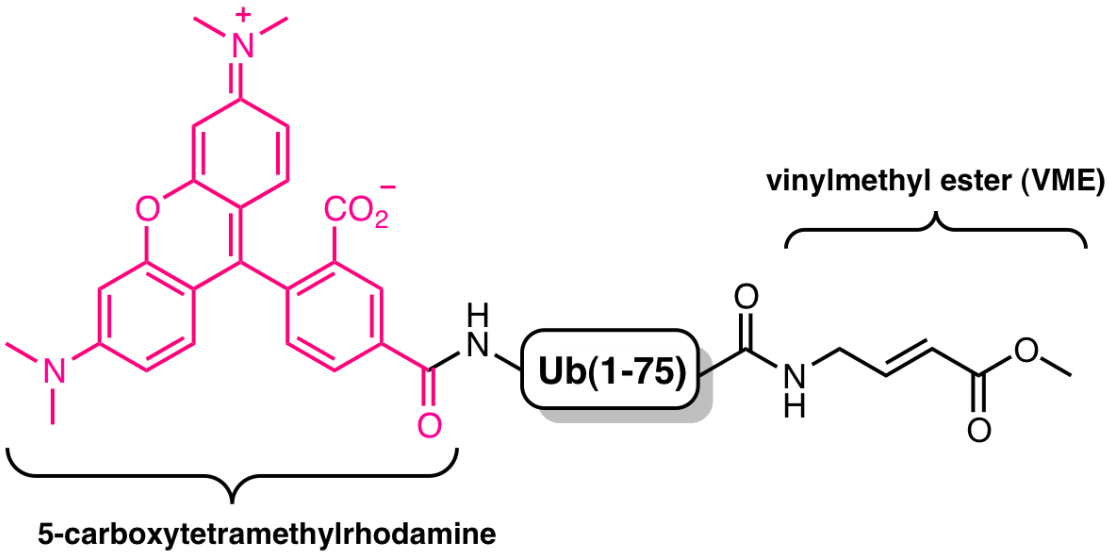



**Figure 2.5.** Chemical structure of the TAMRA-Ub-VME probe designed for Gel-based labeling of DUBs. Pink colored structure indicates fluorogenic TAMRA (5-carboxytetramethylrhodamine) dye.

### 2.6.5.2. Ubiquitin-Rhodamine (Ub-Rho) assay

Ubiquitin-Rhodamine assay was performed in 20 mM Tris-HCl, 100 mM NaCl at pH 7.5, 5 mM DTT, 0.5 mg/ml BGG (Bovine Gamma Globulin) and 10 mg/ml CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate) with 25 nM USP32-FL and 6.25 nM USP32-CD enzyme concentrations depending on their relative activity. Finally, Ub-Rho substrate (100 nM) was added into the reaction tubes. Cleavage of the amino bond between Ub and Rho by enzymatic activity was measured at 485 nM excitation and 535 nM emission filters using PerkinElmer Wallac EnVision 2010 Multilabel Reader. Cleavage site of the Ub-Rho is demonstrated in Figure 2.6.

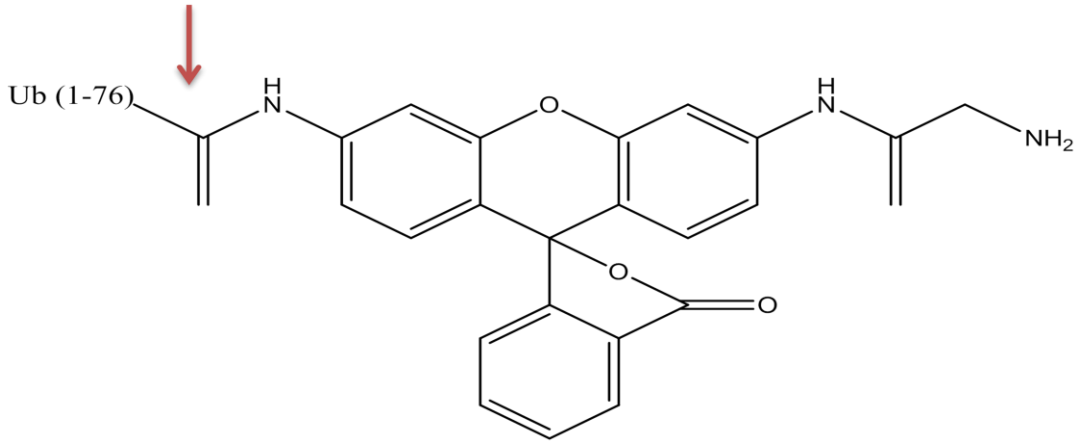



**Figure 2.6.** The cleavage of the amide bond between the C-terminal glycine of ubiquitin and rhodamine.

### 2.7. Diubiquitin (Di-Ub) hydrolysis assay

Di-Ub hydrolysis assay was performed in 50 mM Hepes buffer (pH:7.5), 100 mM NaCl, 5 mM dithiothreitol (DTT) buffer with 75 nM enzyme concentration (USP32-FL and USP32-CD) and 5 µg Di-Ub with specific linkage from Netherlands Cancer Institute (NKI) (Linear, K6, K11, K27, K29, K33, K48 and K63 Di-Ubs) for each reaction. Hydrolysis reactions were kept at 37 $^{o}$C for 0, 30 and 60 min. Reactions were then stopped by adding the sample loading buffer and consequent boiling. Samples were loaded and run on 4-12 % Bis-Tris NuPage gel (Invitrogen). Gel was stained with coomassie blue and imaging was performed using molecular imager ChemiDoc XRS+system with image lab software (BioRad).

### 2.8. Kinetic analysis with Ub-FP hydrolysis assay

Ub-FP assay was performed with different concentration of USP32-FL and USP32-CD to determine suitable enzyme concentration. 9 different concentrations of USP32-FL and USP32-CD (100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.1 nM, 1.6 nM, 0.8 nM and 0.4 nM) were used. After determination of enzyme concentration,

Ub-FP assay was performed for different concentration of substrate under a constant enzyme condition. Diluted enzymes were prepared with reaction buffer (20 mM Tris-HCl, 100 mM NaCl at pH 7.5 with 5 mM DTT, 0.5 mg/ml BGG, and 10 mg/ml CHAPS) and different concentrations of substrate (TAMRA K (Ub) G) were prepared with same buffer (2000 nM- 52 nM). After preparation of enzyme and buffer, reaction was started by adding substrate. Ub-FP assay was performed on PerkinElmer Wallac EnVisin2010 multilabel reader with 531nm excitation and 579 nm emission filters. ‘‘Non binding surface flat bottom low flange” black 384 well plate was used for the assay. From the obtained polarization value (P), the amount of processed substrate (Pt) was calculated with the following equation, S=S0-S0x ((Pt-Pmin)/ (Pmax-Pmin)) [153]. S is the amount of processed substrate. S0 is the amount of substrate added to the reaction. Pt is polarization measured from USP32-FL or USP32-CD and substrate reaction in the time point. Pmax is polarization measured from only substrate reaction in the time point Pmin is polarization measured from only control peptide which is corresponding to the deubiquitinated substrate (TAMRA-KG). All data analysis was performed using Microsoft excel and GraphPad prism5 software.

# CHAPTER III

# RESULTS and DISCUSSION

## 3.1. Prediction of conserved domains and catalytic core of USP32

At least 93 deubiquitinating enzymes (DUBs), belonging to 6 families, are encoded from human genome. These enzymes are grouped according to conserved sequences and structures. Some of DUBs have been already identified as active enzymes including USP7 (HAUSP)[154], USP11[155], USP43[156], ect but some others like USP32 remains as predicted DUB enzymes.

Bioinformatics tools (National Center for Biotechnology information (NCBI) Conserved Domain Tools and ExPASy bioinformatic resource portal-Simple Modular Architecture Research Tool (SMART)) suggested that USP32 has C-terminal Cys, His and Asp domains that are common in deubiquitinating enzymes (Figure 3.1). In addition, a comparison of amino acid sequences of known DUBs (USP6, DOa4) revealed conserved residues that coincide with the Cys-His domains (Figure 3.2).

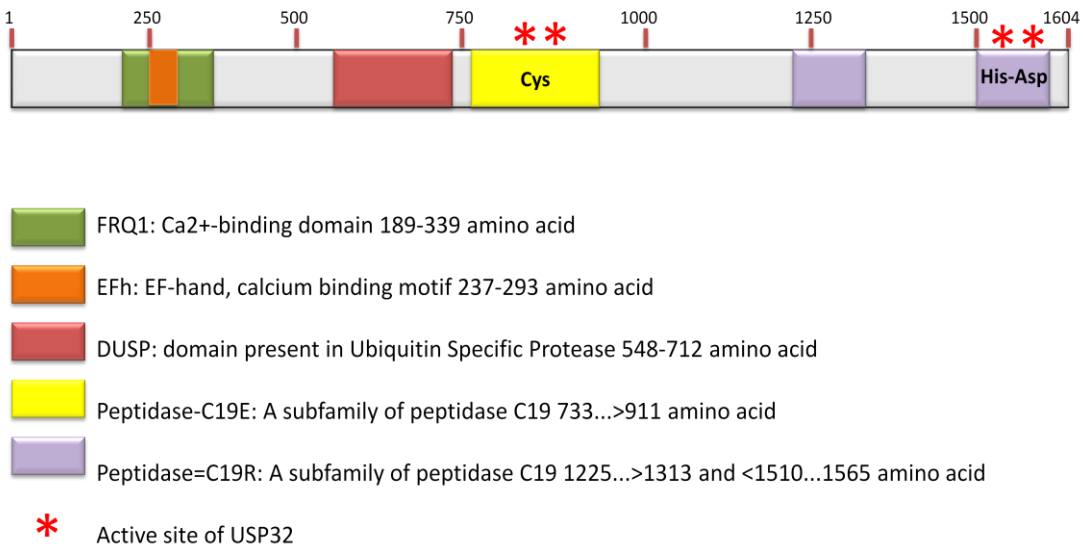



**Figure 3.1.** Schematic outlines of conserved domains of USP32. NCBI and ExPASy-SMART tools predicted USP32 to harbor 5 different regions containing Cys, His and Asp domains which are common for DUBs. $Ca^{+2}$ binding domain and EF-hand motif are located at 189-339 and 237-293 amino acids (aa) positions respectively. DUSP domain (548-712 aa) is found in Ubiquitin specific proteases. 3 peptidase domains are placed at 733-911, 1225-1313 and 1510-1565 aa positions.

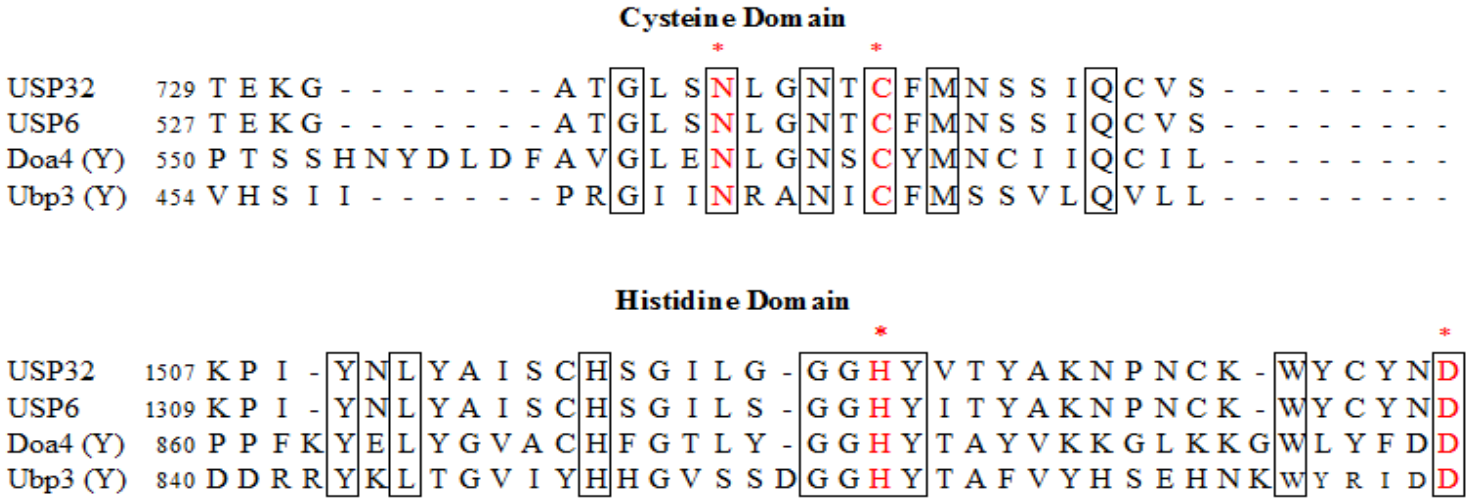



**Figure 3.2.** Sequence alignments of 3 proteins which are engaged in similar functions (deubiquitination) are compared with the amino acid sequence of USP32 by using NCBI Protein Blast and Conserved Domain tools. Asterix marks the active sites of those proteins. Results show that USP32 has the same conserved domains, implicating that it may be involved in deubiquitination functions. (Y) : Yeast Protein.

### 3.2. *In vivo* deubiquitinating enzyme assay

USP32 is predicted to function as a deubiquitinating (DUB) enzyme based on the presence of Cys and His domains commonly found in active deubiquitinating enzymes. Therefore to investigate if USP32 is an active DUB, we performed an *in vivo* deubiquitinating enzyme assay. This assay is based on the co-expression the enzyme to be tested with a ubiquitin (Ub) fusion protein (Ub-met-β-gal) in bacteria. Depending on the activity of the enzyme, Ub is removed from the fusion protein which can be detected by immunoblotting. For this aim, we cloned USP32 into an expression vector (pGEX-4T-2) and investigated its enzymatic activity.

#### 3.2.1. Cloning of *USP32* into GST fusion protein expression vector

To investigate the deubiquitination activity of USP32 and determine the active site, we cloned overlapping fragments of USP32 coding sequence (USP32-I, USP32-II and USP32 III) corresponding to its predicted catalytic core domain (1500-4815 bp) into pGEX-4T-2 GST vector (Appendix B). The position of primers for PCR amplification of *USP32* fragments are given in Figure 3.3.

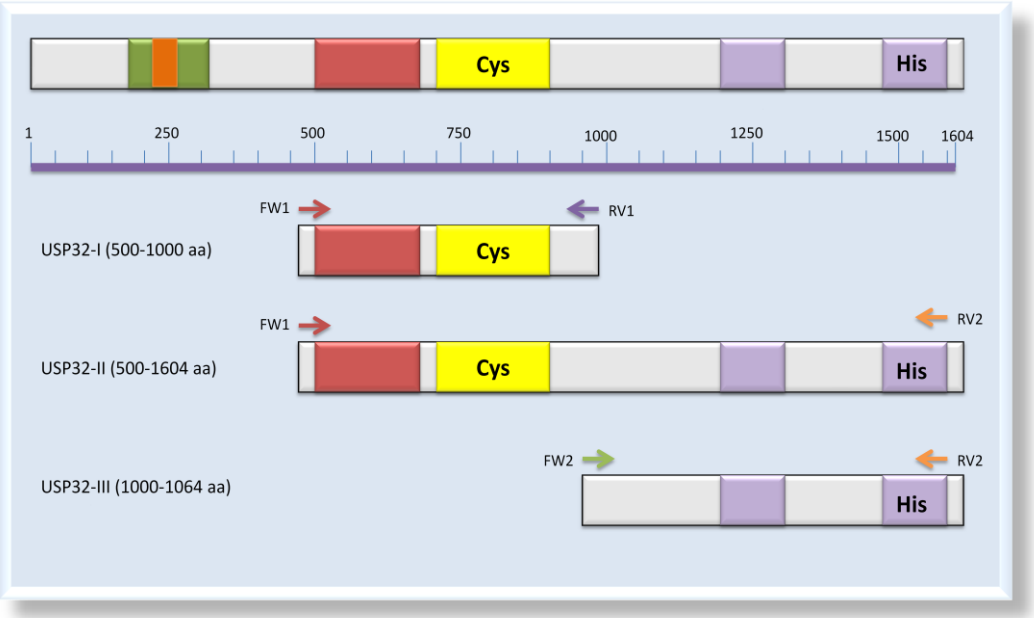



**Figure 3.3.** Positions of cloning primer pairs used for overlapping fragments of USP32 gene. USP32-I (1500-3000 bp), USP32-II (1500-4815 bp) and USP32-III (3000-4815). Primer sequences are available in Appendix A.

*USP32*-I coding sequence was amplified with specific forward and reverse primers containing *SalI* and *NotI* restriction enzyme recognition. After PCR reaction, expected PCR product was obtained (1.8 kb) (Figure 3.4).

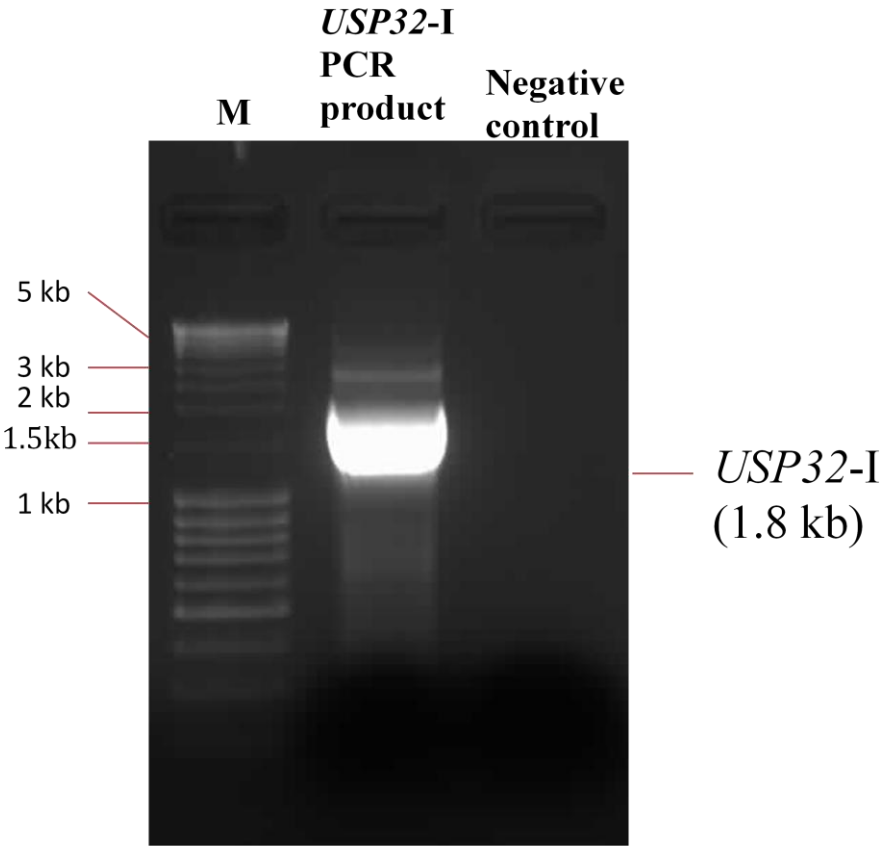



**Figure 3.4.** PCR of USP32-I coding sequence using specific primers containing SalI and NotI restriction enzyme sites. Expected size of *USP32-I* PCR product is 1.8 kb. M: MassRuler DNA ladder (Fermentas, Appendix D).

The PCR product and pGEX-4T-2 vector were both digested with *SalI* and *NotI* restriction enzymes and ligated into pGEX-4T-2 GST fusion protein expression vector (Figure 3.5 A and B). Double digested *USP32*-I PCR product and pGEX-4T-2 vector were exctrated from agarose gel after confirming expected size on gel electrophoresis and ligation reactions were established and performed to obtain GST-USP32-I construct. This construct was transformed into *E. coli* strain Top10. Colonies carrying the correct construct were selected with ampicillin. 5 colonies carrying the GST-USP32-I construct were selected and cloning was confirmed by SalI and *NotI* restriction enzyme digestion (Figure 3.6).

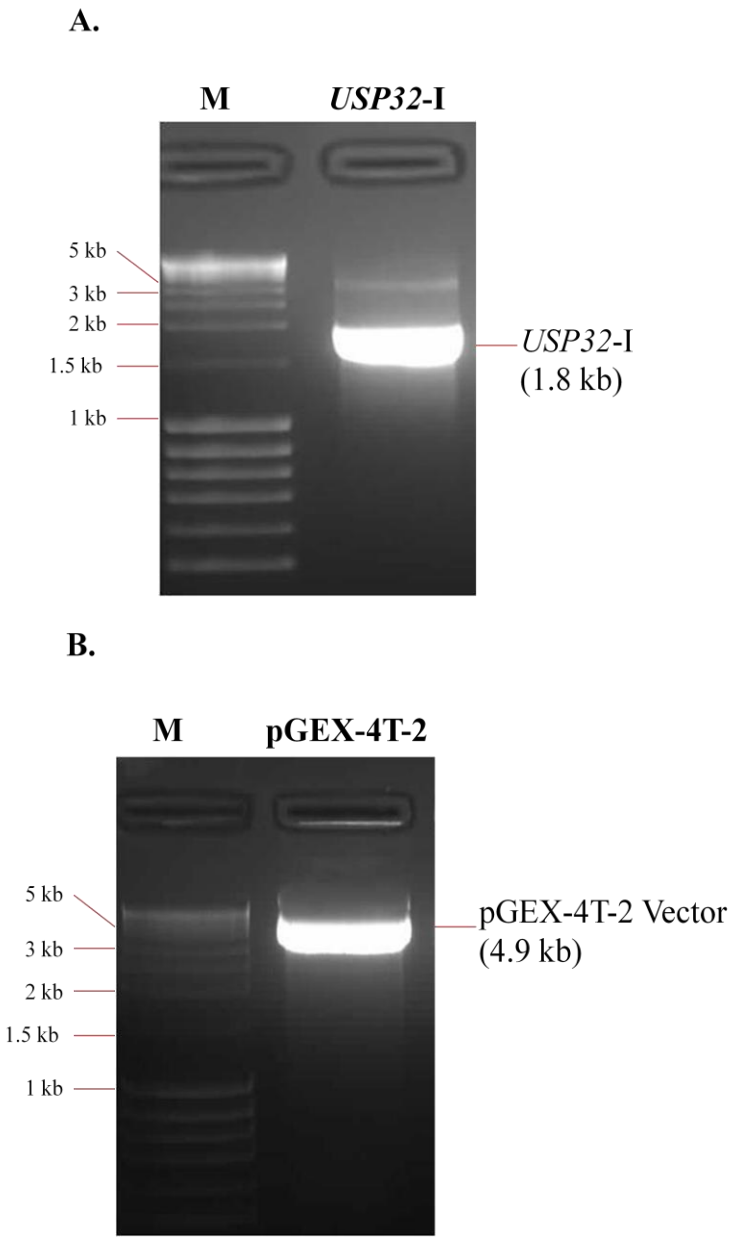



**Figure 3.5.** Double digestion of USP32-I PCR product and pGEX-4T-2 vector with SalI and NotI restriction enzymes. **A.** Double digestion of *USP32*-I PCR product with *SalI* and *NotI* restriction enzymes. **B.** Double digestion of pGEX-4T-2 vector with *SalI* and *NotI* restriction enzymes. **M:** MassRuler DNA ladder (Fermentas, Appendix D).

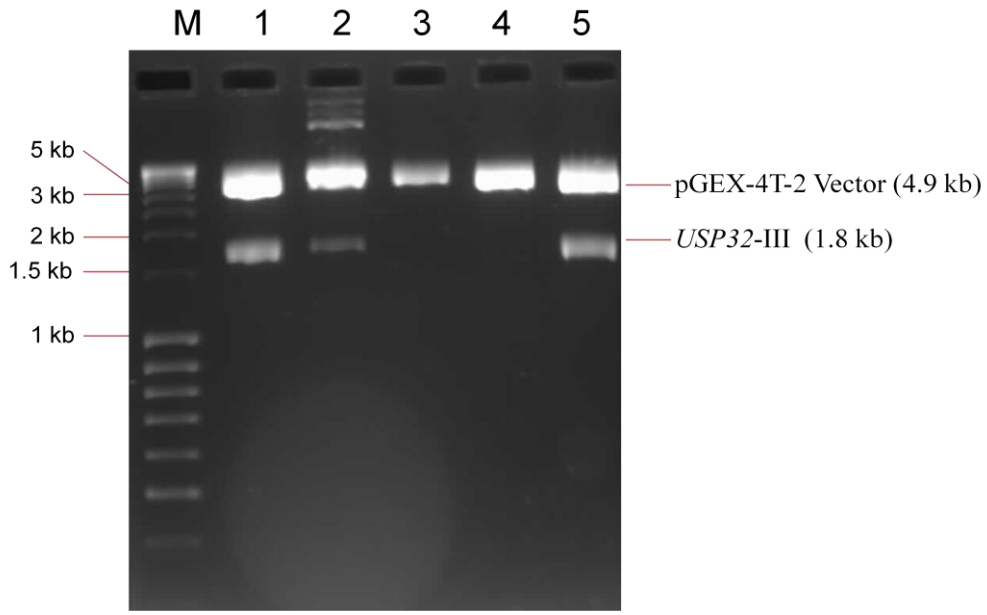



**Figure 3.6.** Restriction digestion confirmation of GST-USP32-I constructs. **M:** MassRuler DNA ladder (Fermentas, Appendix D)., **lane 1**: Colony 1 GST-USP32-I construct digested with *SalI* and *NotI* restriction enzymes, **lane 2**: Colony 2 GST-USP32-I construct digested with *SalI* and *NotI* restriction enzymes, **lane 3**: Colony 3 GST-USP32-I construct digested with *SalI* and *NotI* restriction enzymes, **lane 4**: Colony 4 GST-USP32-I construct digested with *SalI* and *NotI* restriction enzymes, **lane 5**: Colony 5 GST-USP32-I construct digested with *SalI* and *NotI* restriction enzymes. Expected size: 4.9 kb and 1.8 kb.

GST-USP32-I construct was used for further expression experiments after sequence confirmation.

To express GST-USP32-II fusion protein and examine its possible deubiqutination activity, we cloned *USP32-II* fragment corresponding to catalytic core of USP32 into pGEX-4T-2 vector. *USP32-II* coding sequence was amplified with forward and reverse primers containing *SalI* and *NotI* restriction enzyme sites and then PCR product was cloned into pCR8/GW/TOPO entry vector which was double digested with *SalI* and *NotI* (Figure 3.7 A and B). Cloning was confirmed by sequencing.

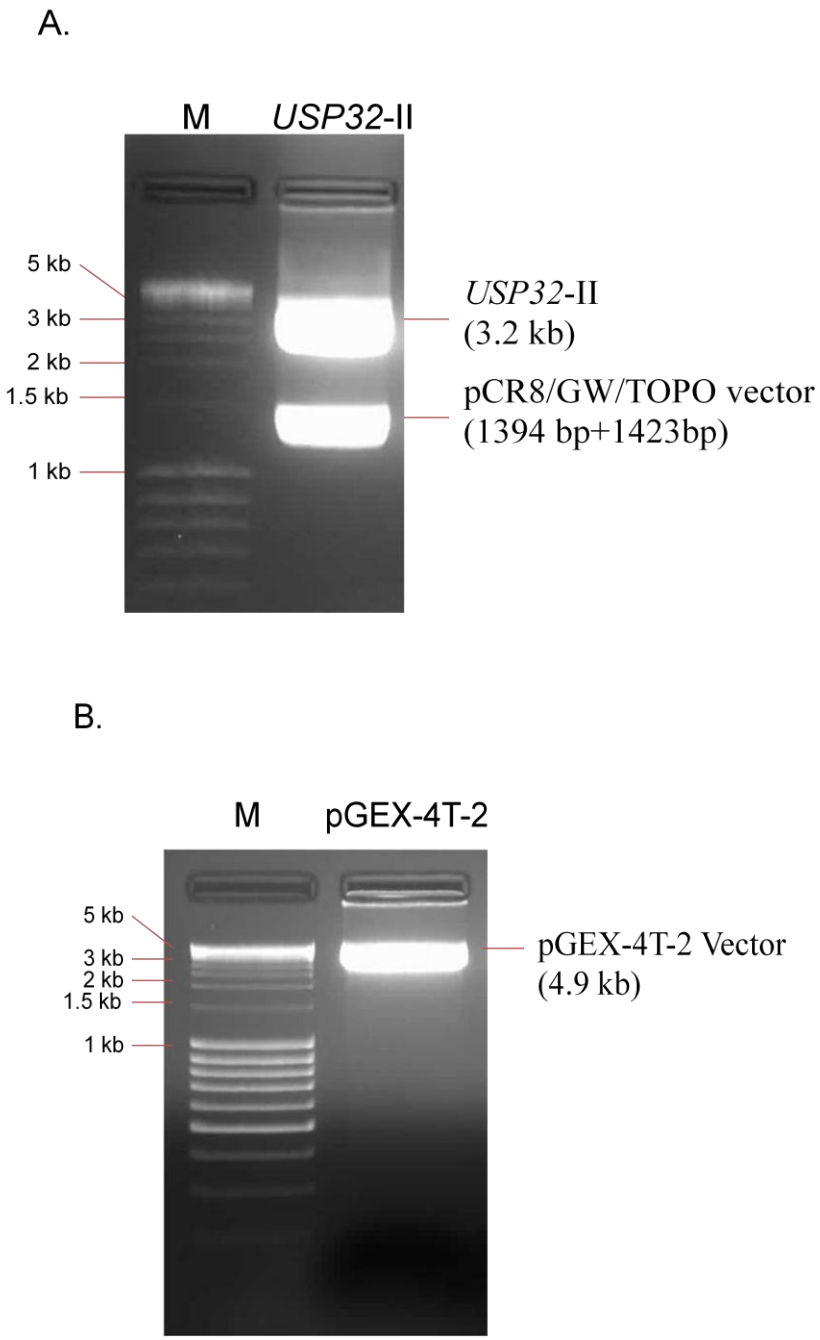



**Figure 3.7.** Double digestion of pCR8/GW/TOPO-USP32-II and pGEX-4T-2 vector with SalI and NotI restriction enzymes. **A.** Double digestion of pCR8/GW/TOPO-*USP32-II* with *SalI* and *NotI* restriction enzymes, resulting in 3.2 kb *USP32-II* fragment and 1394 bp + 1423 bp fragments of pCR8/GW/TOPO vector. **B.** Double digestion of pGEX-4T-2 vector with *SalI* and *NotI* restriction enzymes, generating 4.9 kb linear vector. **M:** MassRuler DNA ladder (Fermentas, Appendix D).

Double digested *USP32-II* fragment and pGEX-4T-2 vector were extracted from agarose gel. Ligation reactions were performed. GST-USP32-II construct was transformed into an *E. coli* strain Top10. 4 colonies carrying correct construct were used to isolate GST-USP32-II construct and the constructs were confirmed with SalI and *NotI* restriction enzymes digestion (Figure 3.8).

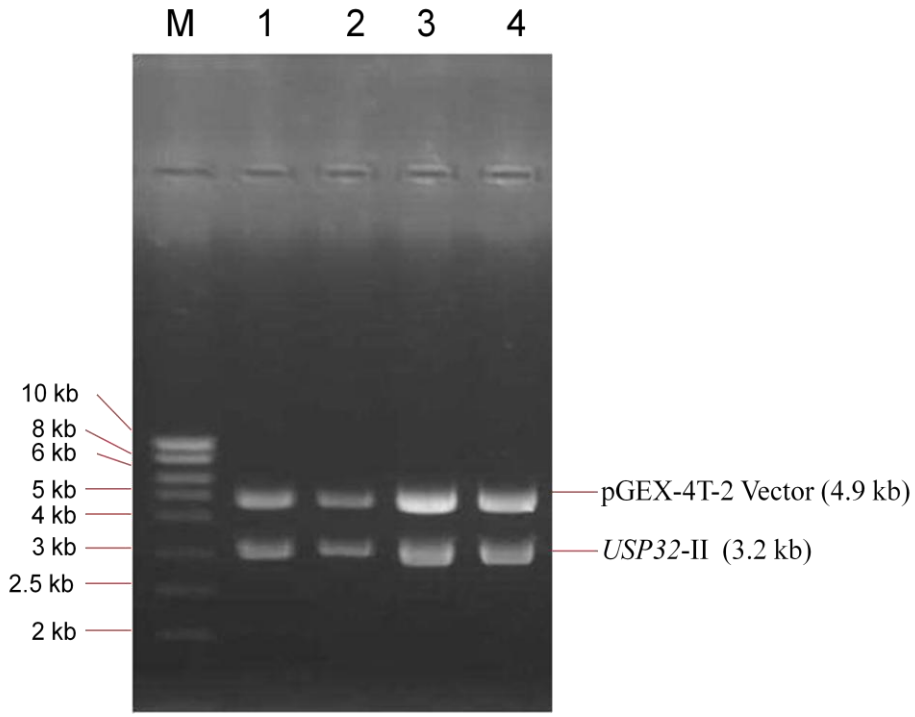



**Figure 3.8.** Restriction digestion confirmation of GST-USP32-II constructs. **M:** MassRuler DNA ladder (Fermentas, Appendix D)., **lane 1**: Colony 1 GST-USP32-II construct digested with *SalI* and *NotI* restriction enzymes, **lane 2**: Colony 2 GST-USP32-II construct digested with *SalI* and *NotI* restriction enzymes, **lane 3**: Colony 3 GST-USP32-II construct digested with *SalI* and *NotI* restriction enzymes, **lane 4**: Colony 4 GST-USP32-II construct digested with *SalI* and *NotI* restriction enzymes. Expected size: 4.9 kb and 3.2 kb.

GST-USP32-II construct was used for further expression experiments after sequence confirmation.

Finally, we cloned *USP32-III* coding sequence into GST fusion protein expression vector to investigate whether USP32-III protein has deubiquitination activity. *USP32*-III coding sequence was amplified with specific forward and reverse primers containing *SalI* and *NotI* restriction enzyme recognition sites. After PCR reaction, expected PCR product was extracted from agarose gel (Figure 3.9). The PCR product and pGEX-4T-2 vector were digested with *SalI* and *NotI* restriction enzymes to perform ligation into pGEX-4T-2 vector (Figure 3.10 A and B).

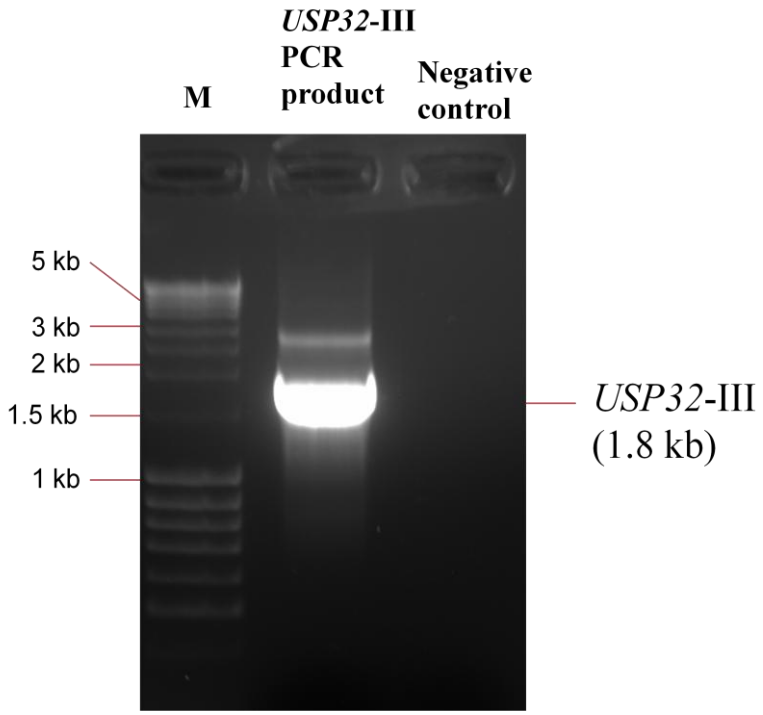



**Figure 3.9.** PCR of USP32-III coding sequence using specific primers containing *SalI* and *NotI* restriction enzyme sites. Expected size of *USP32-III* PCR product was 1.8 kb. **M:** MassRuler DNA ladder (Fermentas, Appendix D).

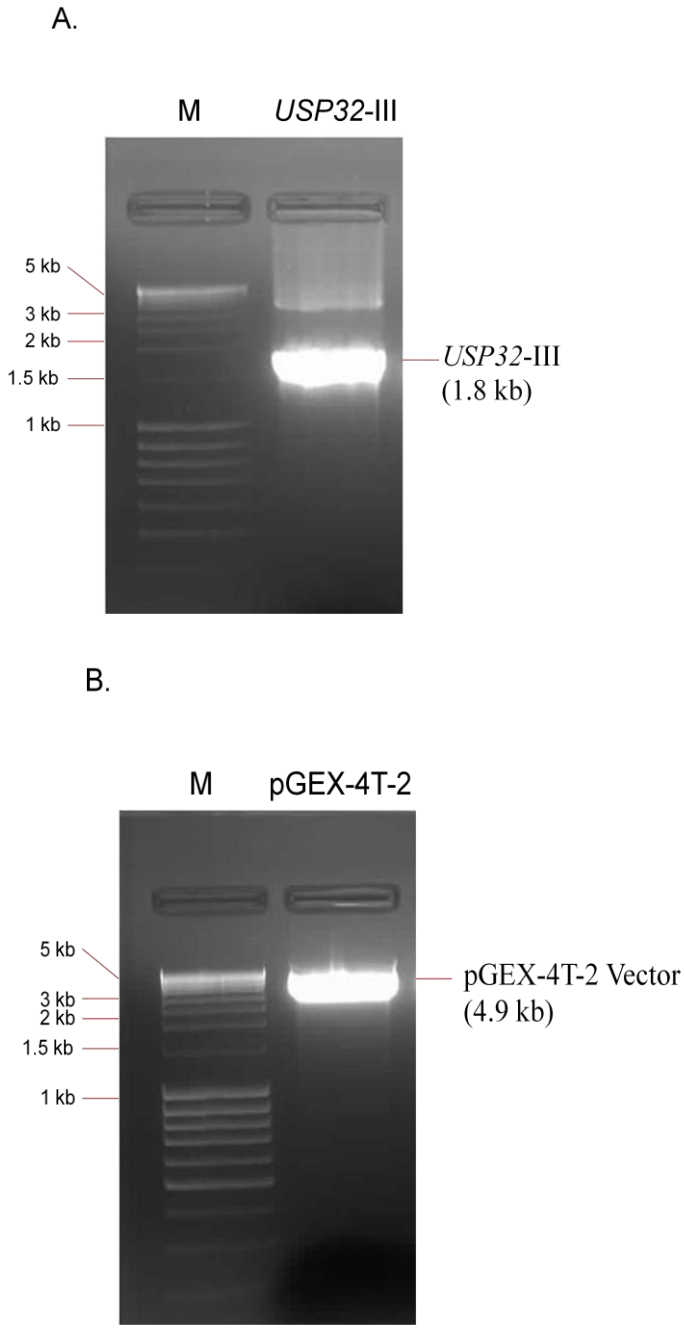



**Figure 3.10.** Double digestion of USP32-III PCR product and pGEX-4T-2 vector with *Sal I* and *Not I* restriction enzymes. **A.** Double digestion of *USP32*-III PCR product with *SalI* and *NotI* restriction enzymes, resulting in a 1.8 kb product. **B.** Double digestion of pGEX-4T-2 vector with *Sal I* and *Not I* restriction enzymes, resulting in a 4.9 kb linear vector. **M:** MassRuler DNA ladder (Fermentas, Appendix D).

Double digested *USP32-III* fragment and pGEX-4T-2 vector were extracted from agarose and ligation reactions were performed. GST-USP32-III construct was transformed into an *E. coli* strain Top10. 5 Colonies carrying the correct construct were used to isolate GST-USP32-III construct and the constructs were confirmed with *Sal I* and *Not I* restriction enzymes digestion (Figure 3.11).

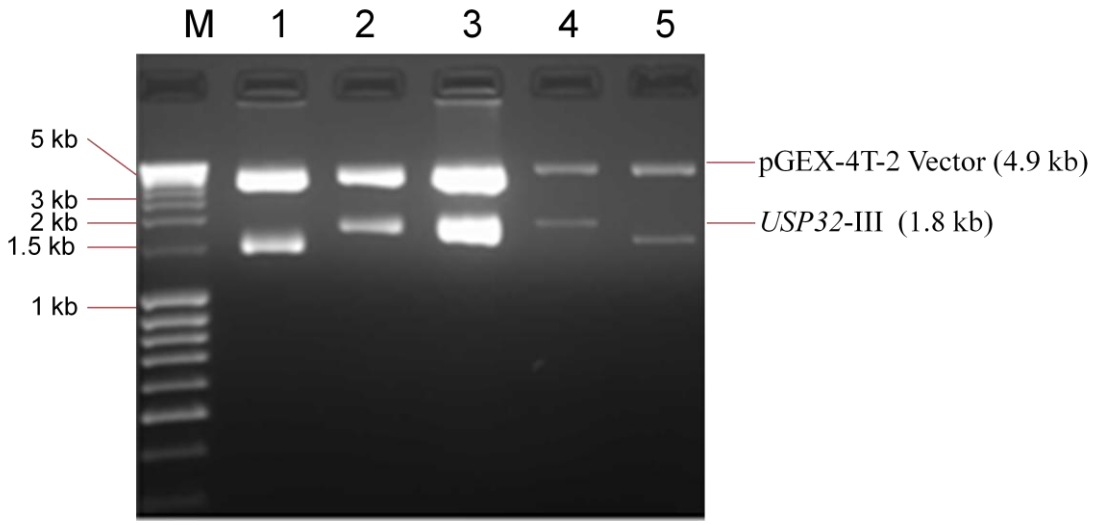



**Figure 3.11.** Restriction digestion confirmation of GST-USP32-III constructs with *Sal I* and *Not I* restriction enzymes. **M:** MassRuler DNA ladder (Fermentas, Appendix D), **lane 1**: Colony 1 **lane 2**: Colony 2 **lane 3**: Colony 3, **lane 4**: Colony 4, **lane 5**: Colony 5. Expected size: 4.9 kb and 1.8 kb.

As a result of all cloning experiments, we obtained 3 correct constructs that harbor different regions of USP32. These construct were then tested for deubiquitinating enzyme activity.

### 3.2.2. Investigation of deubiquitination activity of USP32

USP32 protein has 5 domains that contain 2 catalytic domains (Cys and His ) in addition to calcium binding and DUSP domains as mentioned in Figure 3.1 and Figure 3.2. Cys and His domains are common in all deubiquitinating enzymes.

Therefore, we hypothesized that USP32 protein may be an active deubiquitinating enzyme.

To investigate and confirm this, we initially performed an *in vivo* deubiquitinating enzyme assay. The deubiquitination assay is based on the cleavage of ubiquitin–beta-galactosidase (ub-β-gal) fusion proteins. If USP32 acts as deubiquitinating enzyme, we expected it to remove Ub from Ub-met-β-gal. The positive control, UBP3, containing highly conserved Cys and His domains is known to be able to remove Ub from Ub-met-β-gal [78].

For the *in vivo* deubiquitinating enzyme assay [78, 148, 154], PACYC184-Ub-met-β-gal construct and pGEX-4T-2-*USP32*-I, pGEX-4T-2-*USP32*-II and pGEX-4T-2-*USP32*-III containing *USP32* fragments and pGEX-*UBP3* constructs were co-transformed into DH5α *E.coli*. PGEX-*UBP3* (a gift from Dr. M.Hochstrasser) was used as positive control for deubiquitinating enzyme activity. UBP3 was earlier reported as an active deubiquitinating enzyme [78]. Colonies that contain PACYC184-Ub-met- β-gal and GST fusion construct were selected by using LB media with 100 µg/ml of ampicillin and 10 µg/ml of chloramphenicol. Plasmid-bearing *E.coli* DH5α cells were grown in 2X YTA media until $A_{600}$ reached 0.6-0.8. Isopropyl-1-thio-ß-Dgalactopyranoside (IPTG) was then added to a final concentration of 0.2 mM. After 4h of incubation with IPTG at 37 $^{o}$C, cells were lysed in 1XPBS buffer containing 100 µg/ml Lysozyme, protease inhibitor (Roche Complete Mini Protease Inhibitor Cocktail Tablets in 10ml 1XPBS buffer) and 10U/ml DNase I by using freeze/thaw cycles [149].

To verify GST fusion protein expression in DH5α *E.coli*, total proteins from pGEX-*USP32*-I, pGEX-*USP32*-II, pGEX-*USP32*-III, pGEX-*UBP3* as well as negative controls (Ub-beta-gal and Ub-beta-gal + pGEX (empty vector) were loaded on 10% SDS gels.- Expression of GST-USP32 fusion protein was verified by immunoblotting (Figure 3.12)

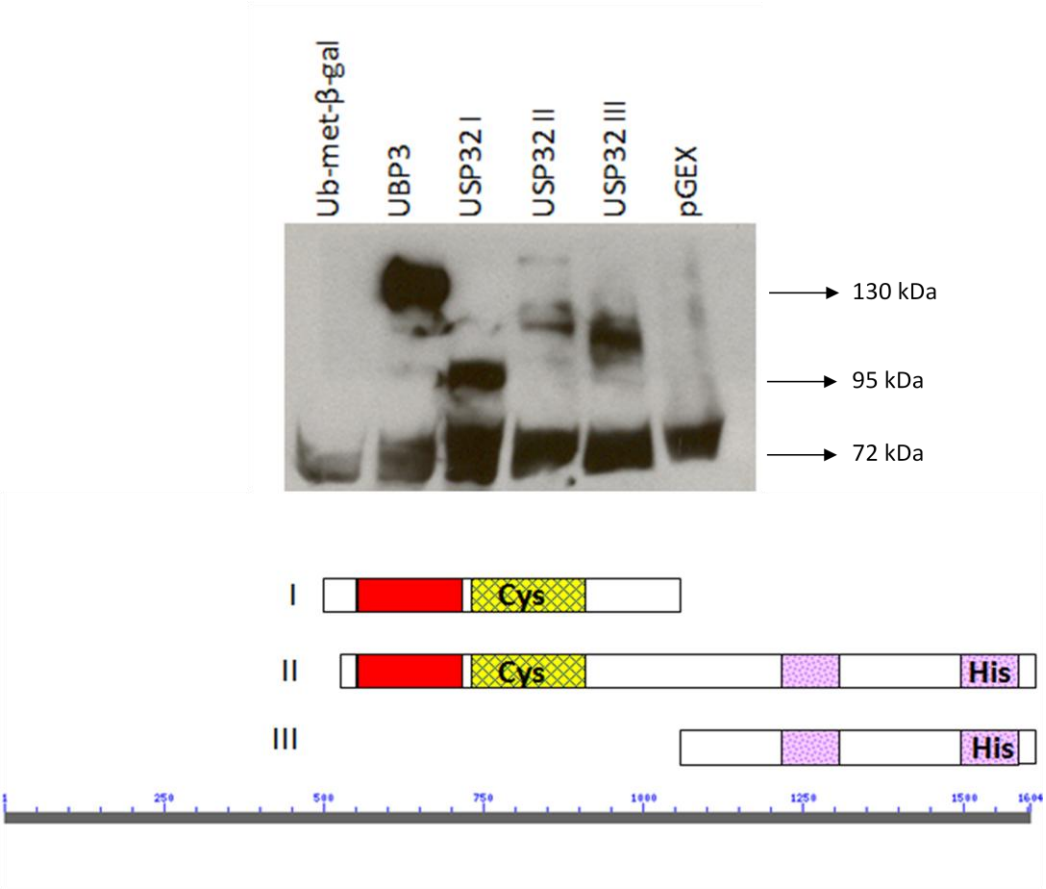



**Figure 3.12.** Western blot analysis of GST fusion proteins expression. The cells were harvested and proteins were subjected to 10% SDS-PAGE, followed by immunostaining with 1:2000 goat anti-GST antibody (Amersham) and 1:10000 Anti-goat-HRP secondary antibody (Santa Cruz). PACYC184-Ub-met-β-gal (lane1), pGEX-UBP3+Ub-met-β-gal (lane2), pGEX-*USP32*-I+Ub-met-β-gal (lane3), pGEX-*USP32*-II+Ub-met-β-gal (lane 4) pGEX-*USP32*-III+Ub-met-β-gal (lane5) and pGEX+ Ub-met-β-gal (Lane 6). Box in red indicates DUSP domain (domain present in Ubiquitin Specific Protease 548-712 amino acid). Boxes in yellow and purple indicate peptidase-C19E (733-911 amino acid) peptidase-C19R (1225-1313 and <1510-1565 aa) domains. Expected sizes for GST-UBP3, GST-USP32-I, GST-USP32-II and GST-USP32-III are 130 kDa, 94.7 kDa, 151.7 kDa and 97.4 kDa respectively.

Figure 3.12 shows expression of GST fusion proteins GST-UBP3, GST-USP32-I, GST-USP32-II and GST-USP32-III detected by a specific GST antibody. We detected GST-UBP3 (positive control) as expected size, 130 kDa. For GST-USP32-I,

we observed one band around 95 kDa which is approximately expected size (94, 7 kDa). We also expectedly detected GST-USP32-II with low expression level because of large size of this protein (151 kDa) and so degradation of protein but its expression is enough for *in vivo* deubiquitination assay. For GST-USP32-III, we detected the expected protein band with some degradation products. Finally, for negative controls, only Ub-β-gal and pGEX+ Ub-β-gal, we detected some background bands. These results showed that all GST fusion proteins were expressed in cotransformed bacteria and they could be used for further *in vivo* deubiquitination assay.

After detecting the GST fusion proteins, to confirm deubiquitinating enzyme activity of USP32 catalytic domains, we used anti-β-galactosidase antibody (Figure 3.13).

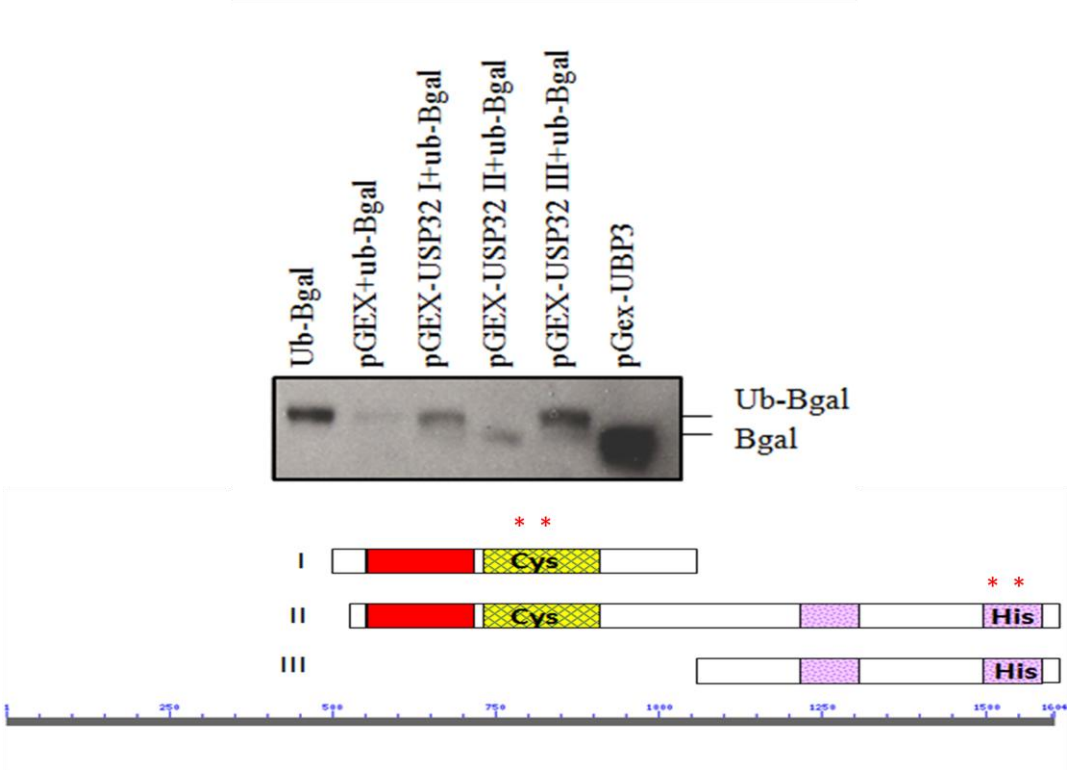



**Figure 3.13.** Result of in vivo deubiquitinating enzyme assay. The cells were harvested and proteins were subjected to 10 %SDS-PAGE, followed by immunostaining with mouse monoclonal β-galactosidase antibody (cell signalling) in 1:1000 dilution and anti- mouse-HRP secondary antibody (Santa Cruz) in 1:2000 dilution-. PACYC184-Ub-met-β-gal (lane1), Ub-met-β-gal+pGEX (lane2), Ub-met-β-gal+ pGEX-USP32-I (lane3), Ub-met-β-gal+pGEX-USP32-II (lane 4) Ub-met-β-gal+pGEX-USP32-III (lane5). Ub-met-β-gal +pGEX-UBP3 (lane6, positive control).

As expected for positive control (GST-UBP3), we detected removal of Ub from the Ub-β-gal fusion protein which yielded a lower sized β-gal band (117 kDa). For the negative control (no USP32 construct), we detected Ub-β-gal (125 kDa) because Ub was not be removed from Ub-β-gal. For USP32-II which includes all three active peptidase domains, we detected removal of Ub-β-gal and a lower sized β-gal (117 kDa) was observed similar to the positive control; UBP3. For USP32-I (contains only one peptidase region corresponding to 733->911 amino acid) and USP32-II

(harbors two peptidase regions corresponding to1225->1313 and <1510-1565 amino acid), we detected Ub-β-gal (125 kDa) like as negative control. These results suggested USP32 to be an active enzyme and that Cys, His and Asp domains are required for the enzymatic activity.

### 3.3. Biochemical analysis of USP32

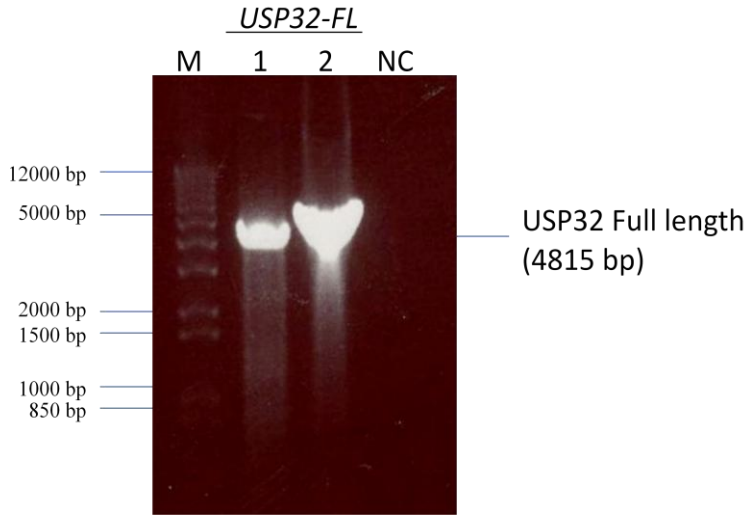
To further investigate biochemical properties of USP32, we cloned and expressed USP32 protein using an insect system (pFastNKI-his3C-LIC) Enzymatic activity of purified protein was confirmed using *in vitro* deubiquitinating enzyme assay as described below. Active enzyme was used for further analysis (detection of Ub-linkage specifity, kinetic analysis of USP32 and inhibitor screening)

#### 3.3.1. Cloning of full length USP32 into pFastNKI-his3C-LIC

To further confirm the enzymatic activity, we expressed and purified USP32 Full length (FL) and catalytic domain (corresponds to Cys and His domains) (CD) proteins. For this purpose, *USP32-FL* (1-4815 bp) and *USP32-CD* (2160-4815 bp) were cloned into pFastNKI-his3c-LIC vector using ligation-independent cloning strategy as mentioned in Chapter 2. *USP32-FL* and *USP32-CD* coding sequences were amplified with specific forward and reverse primers containing vector compatible 5'overhangs (Appendix A) as indicated in Figure 3.14.

pFastNKI-his3c-LIC vector is for insect cell expression and contains 6XHis-Tag to generate His –tagged proteins to facilitate protein purification. Further information on pFastNKI-his3c-LIC vector is given in Appendix B.

**A.**




**B.**

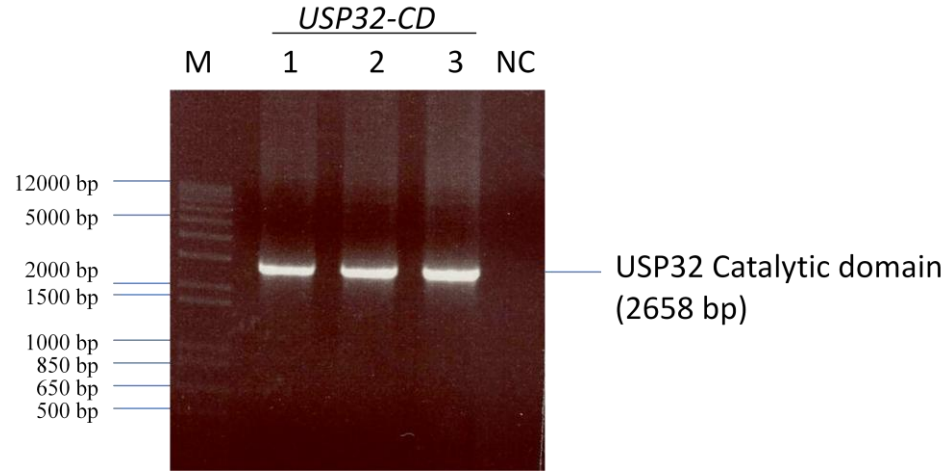
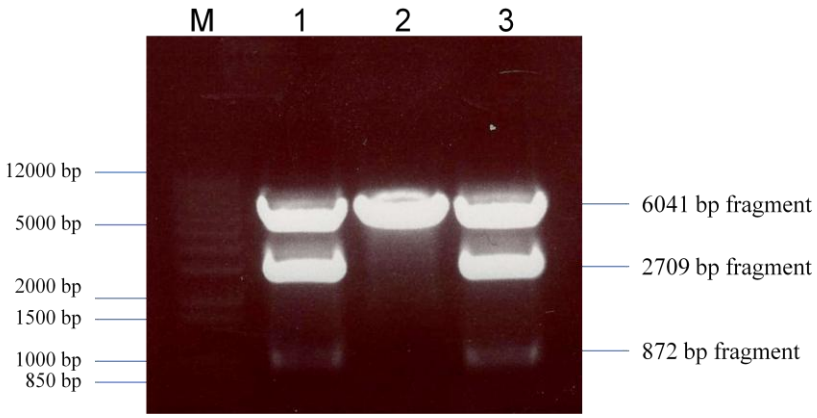



**Figure 3.14.** PCR of USP32-FL and USP32-CD coding sequence with specific primer for LIC cloning strategy. **A.** PCR of *USP32-FL* coding sequence with specific primer for LIC cloning strategy. Expected size of *USP32-FL* PCR product was 4815 bp. **B.** PCR of *USP32-CD* coding sequence with specific primer for LIC cloning strategy. Expected size of *USP32-CD* PCR product was 2658 bp. **NC:** Negative Control (no template control). **M:** 1 Kb plus DNA ladder (Invitrogen, Appendix D).

After amplification of *USP32-FL* and *USP32-CD* coding sequence using PCR method, Agarose extracted PCR products were cloned into pFastNKI-his3c-LIC vector through Ligation Independent Cloning (LIC) strategy as previously mentioned in Figure 2.3. Vector was digested with *Kpn I* restriction enzyme to create blunt-end and then treated with T4 DNA polymerase and dTTP. PCR products were also treated with T4 DNA polymerase and dATP to generate vector compatible 5'overhangs. T4 DNA polymerase treated vector and insert were combined with each other and then transformed into *DH5α E.coli* strain.

After transformation, 3 colonies carrying *USP32-FL* or *USP32-CD* constructs were used for plasmid isolation and *USP32-FL* and *USP32-CD* constructs were verified with *NcoI* restriction enzyme digestion (Figure 3.15).

**A.**




**B.**

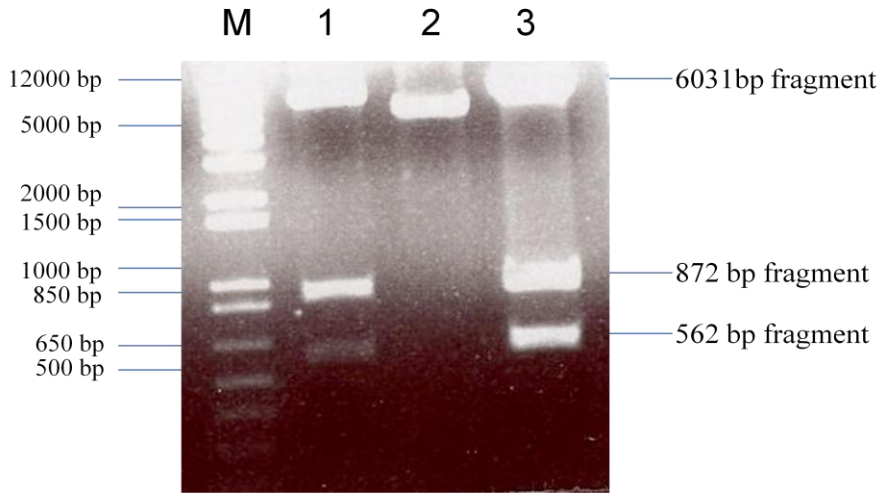



**Figure 3.15.** Restriction digestion confirmation of USP32-FL and USP32-CD constructs with *Nco I* restriction enzyme digestion. **A. lane 1**: Colony 1 *USP32-FL* construct digested with *Nco I* restriction enzyme, **lane 2**: Colony 2 *USP32-FL* construct digested with *Nco I* restriction enzyme, **lane 3**: Colony 3 *USP32-FL* construct digested with *Nco I* restriction enzyme, resulting in 6041 bp, 2709 bp and 872 bp fragments. **B. lane 1**: Colony 1 *USP32-CD* construct digested with *Nco I* restriction enzyme, **lane 2**: Colony 2 *USP32-CD* construct digested with *Nco I* restriction enzyme, **lane 3**: Colony 3 *USP32-CD* construct digested with *Nco I* restriction enzyme, resulting in 6031 bp, 872 bp and 562 bp fragments. **M:** 1 Kb plus DNA ladder ( invitrogen, Appendix D).

All constructs were then verified by DNA sequencing. *USP32-FL* and *USP32-CD* constructs were used for further experiments.

### 3.3.2. Expression and purification of USP32-FL and USP32-CD

To investigate the enzymatic activity and properties of USP32 protein, we expressed USP32 protein in SF9 or SF21 insect cells which were derived from pupal ovarian tissue of the fall armyworm *Spodoptera frugiperda.* We selected this system instead of *E.coli* expression system because in *E.coli,* mammalian proteins may not fold correctly [157]. Additionaly, *E.coli* does not have post-translational modification machinery for mammalian protein expression [158]. *USP32-FL* and *USP32-CD* in pFastNKI-his3c-LIC vector, were transformed into DH10Bac chemically competent cells to screen blue-white colonies and to generate bacmid DNA.

In this system, pFastNKI-his3C-LIC vector contains PolH promoter for high level expression of recombinant protein in insect cells and it is also flanked by Tn7 transposon to enhance generating of recombinant bacmid DNA. Furthermore, the most important component of this system is DH10Bac competent cells. DH10Bac cells carry baculovirus vector (bacmid) with mini-*att*Tn7 site and helper vector to facilitate transposition reaction (Figure 3.16) [159, 160]. Once pFastNKI-his3C-LIC vector construct is transformed into DH10Bac competent cells, transposition reaction will occur and the colonies containing gene of interest are selected by using blue-white screening methods.

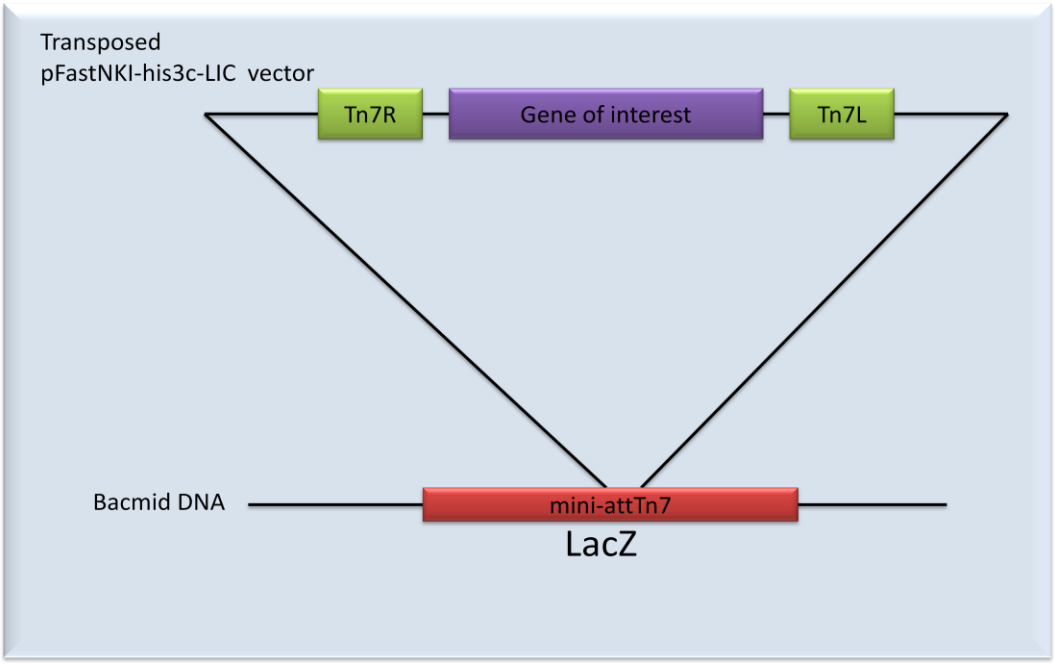



**Figure 3.16.** The strategy of transposition of gene of interest into recombinant bacmid. DH10Bac competent cells contain bacmid DNA with mini-attTn7 site into *LacZ* gene and gene of interest was cloned into pFastNKI-his3c-LIC vector with Tn7 flanking regions in order to enhance transposition reaction.

Cells were spread on LB agar plate and white colonies were selected for bacmid isolation to generate the recombinant baculovirus (Figure 3.17).

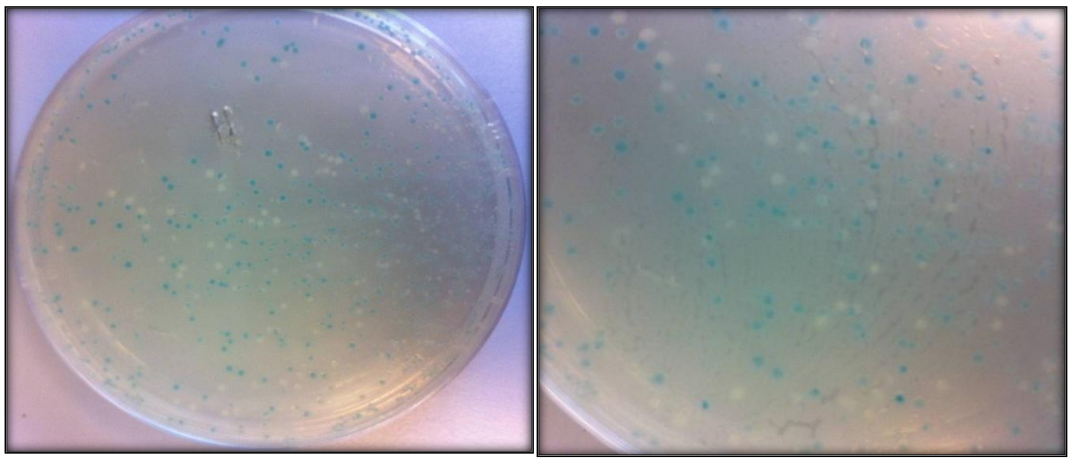


**Figure 3.17.** Selection of pFastNKI-his3C-LIC-USP32 constructs using blue-white screening. Cells were spread on LB agar plate containing 50µg/ml kanamycin, 7µg/ml gentamicin, 10µg/ml tetracycline, 100µg/ml Bluo-gal, and 40µg/ml IPTG. Bluo-gal is a substrate for β-galactosidase. IPTG is an inducer for lac operon and used to enhance LacZ expression.

After screening blue-white colonies, several white colonies were selected for purification of recombinant bacmid DNA. The purified bacmid DNAs were used for transfection of SF9 or SF21 insect cells. Transfected insect cells were incubated until seeing signs of viral infection. Signs of viral infection are increase of cell diameter, size of cell nuclei and decrease of cell growth, granular appearance, detachment and cell lysis when transfected cells are compared to only cells control. These signs are generally monitored 72 hours after transfection. Therefore, once cells represented signs of infection, culture medias were collected as a P1 viral stock.

P1 viral stock was used for small scale protein expression to confirm recombinant protein expression and preparing of P2 viral stock. Cell pellets were used for protein isolation and culture medias were used for P2 viral stock 72 hours after transfection. After P2 viral stock which provides the highest protein expression was selected, 1000 times dilution of P2 viral stock was used for large scale protein expression and purification. After 72 h, media was used as virus stock and insect cells pellet was used for protein isolation as described in materials and methods (Chapter 2) USP32-FL and USP32-CD protein isolates were then run on 4-12% SDS gels and stained

with coomassie blue to confirm protein expression (Figure 3.18 A and B, respectively).

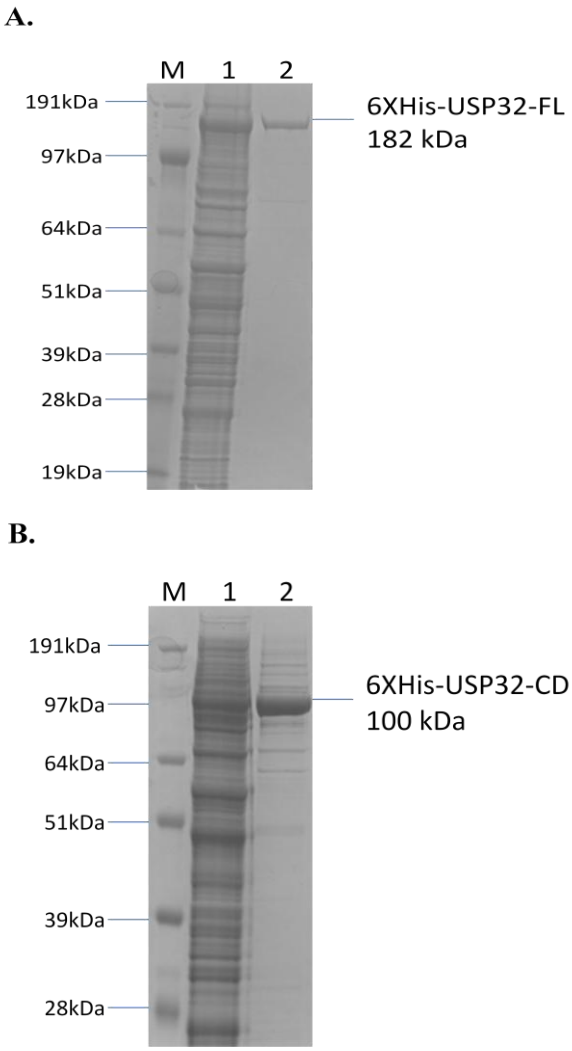



**Figure 3.18.** Protein expression and purification for USP32. **A.** Protein expression and purification for USP32-FL. **M:** SeeBlue® Plus2 Pre-Stained Standard (invitrogen, Appendix D), **lane 1**: Total protein from insect cell infected with baculovirus containing USP32-FL expression construct, **lane 2**: His-tag purified USP32-FL protein (Expected mass: 182 kDa). **B.** Protein expression and purification for USP32-CD. **M:** SeeBlue® Plus2 Pre-Stained Standard (invitrogen, Appendix D), **lane 1**: Total protein from insect cell infected with baculovirus containing USP32-CD expression construct, **lane 2**: His-tag purified USP32-CD protein (Expected mass: 100 kDa).

Purified proteins were run on 4-12%SDS gel (Figure 3.19) and the concentrations of proteins were calculated by NanoDrop.

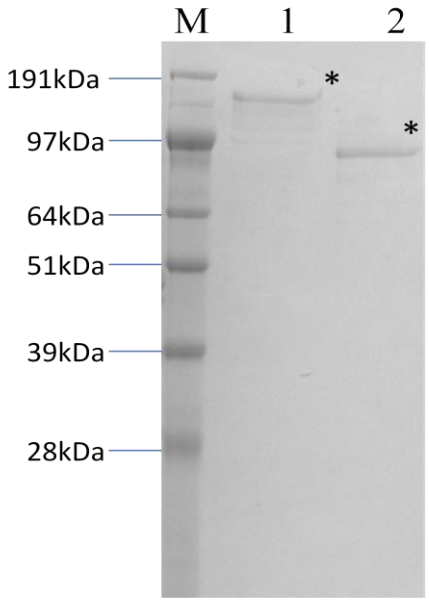



**Figure 3.19.** Final purification product of the USP32-FL and USP32-CD construct run on SDS-PAGE gel. Lane1: SeeBlue® Plus2 Pre-Stained Standard marker from invitrogen (Appendix B), lane 2: USP32-FL protein product (182kDa) and lane 3: USP32-CD protein product (100kDa). Asterisks indicate the expressed USP32-FL and USP32-CD protein. USP32-FL and USP32-CD have N-terminal His tag.

After expressing and purifying of USP32-FL and USP32-CD protein, USP32-FL and USP32-CD protein were used for further *in vitro* experiments.

### 3.3.3 Determination of *in vitro* activity of USP32-FL and USP32-CD via gel based-TMR-Ub-VME (TAMRA-Ubiquitin-Vinyl methyl ester) labeling

To investigate the activity of USP32 and to confirm whether USP32 was the only active DUB in purified protein, we used gel-based TMR-Ub-VME probe labeling strategy. Earlier studies commonly used the HA-Ub-VME probe to detect activity of DUBs including UL36, USP7, FAM, USP15, UCHL-1 and UCHL-3 [161, 162] . However, TAMRA-Ub-VME probe direct detection without immunoblotting methods and eliminates non specific binding.

In this strategy, TMR-Ub-VME probe contains carboxytetramethylrhodamine (TAMRA) dye which is a fluorescent dye derived from rhodamine and is for labeling of ubiquitin and Ub-VME which is a C-terminal derivative of ubiquitin with glycyl vinyl methyl ester instead of Gly76 residue of ubiquitin and a specific inhibitor for cysteine protease DUBs. The basic principle of TMR-Ub-VME is that the sulphur group of active cysteine in DUB attacks the bond between Vinyl methyl ester and ubiquitin. Covalent thioether bond which mimics thioester bond forms between ubiquitin and DUB and so DUB covalently binds to Ub and fluorescent TAMRA dye. Therefore, active DUBs are detected (Figure 3.20).

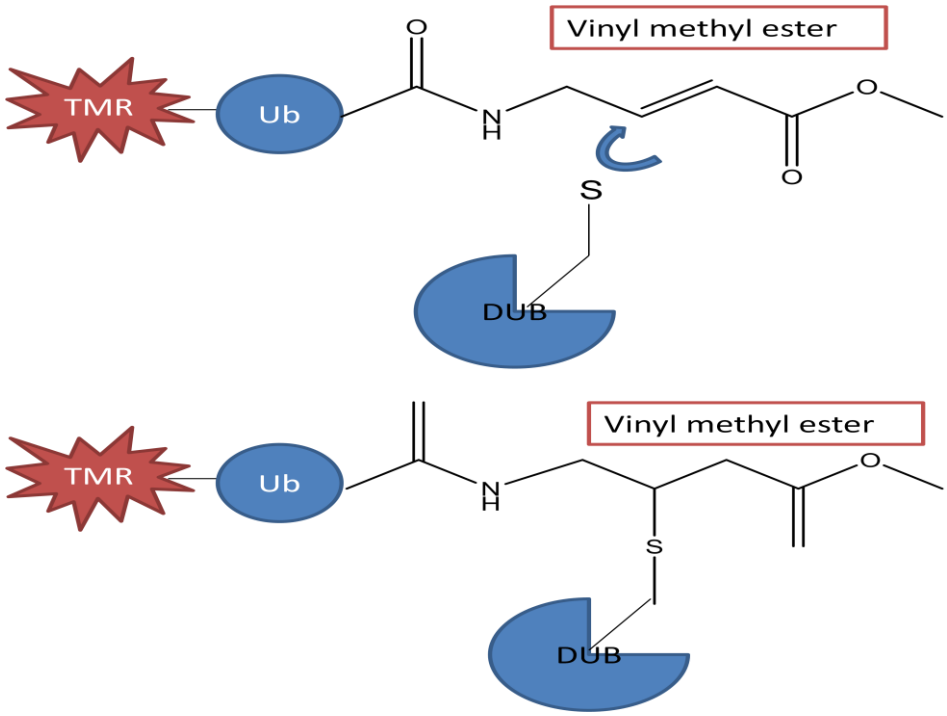
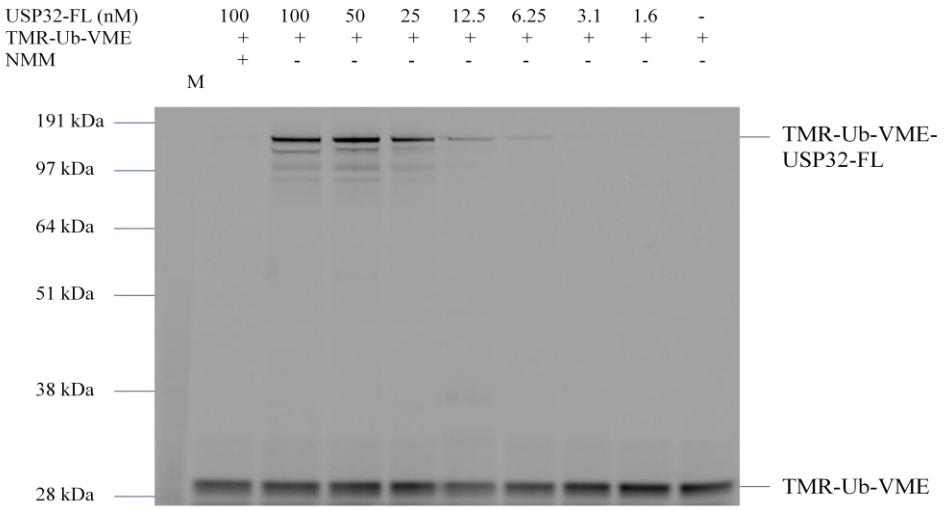



**Figure 3.20.** The basic principle of TMR-Ub-VME is that the sulphur group of active cysteine in DUB attacks the bond between vinyl methyl ester and ubiquitin. Covalent thioether bond forms between ubiquitin and DUB and so DUB binds to Ub and also fluorescent TAMRA (TMR) dye.

**A.**




**B.**

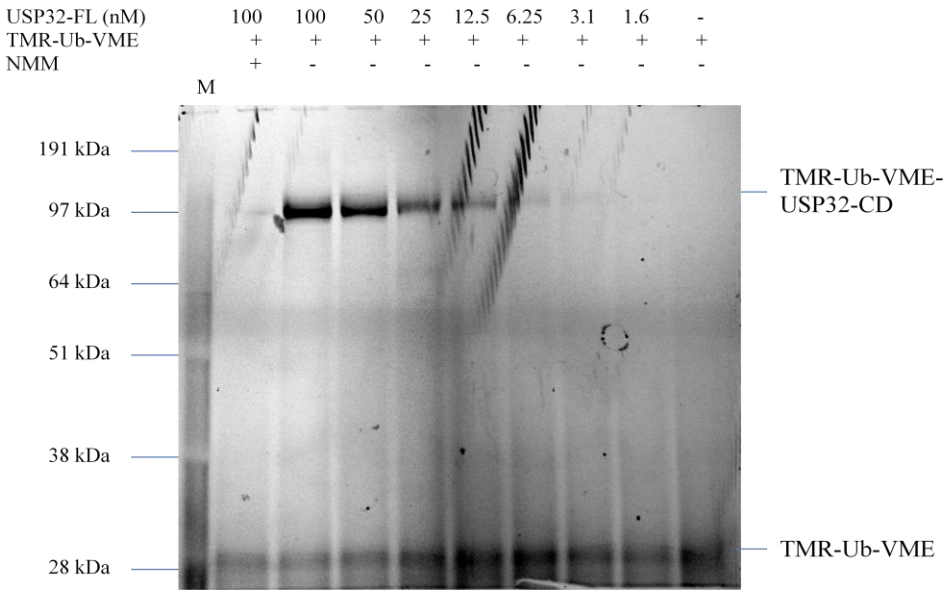



**Figure 3.21.** Identification of activity of USP32-FL and USP32-CD on gel through TMR-Ub-VME. **A.** 7 different concentrations of USP32-FL were used to react with TMR-Ub-VME. Proteins were then separated by 4-12% SDS-PAGE and analyzed by PerkinElmer ProExpress 2D Proteomic Image System using 535 nM excitation and 595 nM emission filters. Reactions were carried out in the absence or presence of NMM to determine cysteine specificity. Probe only reaction was used as control (last lane). **B.** 7 different concentrations of USP32-CD were used to react with

TMR-Ub-VME. Proteins were then separated by 4-12% SDS-PAGE and analyzed by PerkinElmer ProExpress 2D Proteomic Image System using 535 nM excitation and 595 nM emission filters. Reactions were carried out in the absence or presence of NMM to determine cysteine specificity. Probe only reaction was used as control (last lane).

According to these results, purified USP32-FL (1-1604 aa) and USP32-CD (720-1604 aa) were active deubiquitinating enzymes. In Figure 3.21A and B, the bands at the bottom of gel are corresponding to TMR-Ub-VME probe size. In Figure 3.21B, only one band corresponding to TMR-Ub-VME-USP32-CD was detected. On the other hand, USP32-FL protein represented other bands in addition to the TMR-Ub-VME-USP32-FL protein band corresponding to 182 kDa. USP32-FL contains N-terminal 6XHis-tag and catalytic domains of USP32 protein are located at C-terminal of protein. After His-tag protein purification, there were no other bands on SDS gel other than the USP32-FL band (182 kDa). The other detected bands after dialysis were considered to be possible degradation products of N-terminal USP32. In this experiment, we also used NMM DUB inhibitor (Figure 3.21A and B, lane 1). NMM (N-methyl maleimide) is an irreversible and specific inhibitor for cysteine proteases. This inhibitor reacts with thiol group found in active cysteine residue of cysteine proteases DUBs. As expected, we could not observe any band in lane 1 because NMM inhibited the deubiquitinating function of USP32.

### 3.3.4. *In vitro* deubiquitinating enzyme assay through Ubiquitin-Rhodamine (Ub-Rho) assay

To further investigate deubiquitinating enzyme activity of USP32-FL and USP32-CD protein, we performed Ubiqiutin-Rhodamin (Ub-Rho) assay. Ub-Rho is a fluorescent substrate for deubiquitinating enzymes which belong to the ubiquitin C-terminal hydrolase (UCH) and ubiquitin specific protease (USP) classes. These deubiquitinating enzymes cleave the amide bond between the C-terminal glycine of ubiquitin and rhodamine. As a result, Rhodamine fluorescence signal measured

using 485nM excitation and 535nM emission filters increases [163]. Ub-AMC (Ubiquitin with 7-amino-4-methylcoumarin) substrate can also be used to determine activity of DUB enzymes as mentioned in previous studies [164, 165]. However, excitation wavelength of AMC overlaps with the UV range and may result in false positive signals [163]. Therefore, use of Ub-Rho substrate was preferred in this study instead of Ub-AMC.

For the DUB activity assay, gradient concentrations of USP32-FL and USP32-CD were used (100 nM, 50 nM, 25 nM, 12.5 nM, 6.25 nM, 3.12 nM, 1.56 nM, 0.78 nM) to determine the catalytic activity of USP32-FL and USP32-CD. Following the addition of Ub-Rhodamine (100 nM), fluorescence signals were quantified using a microplate reader every 2 minutes for 1.5 hours.

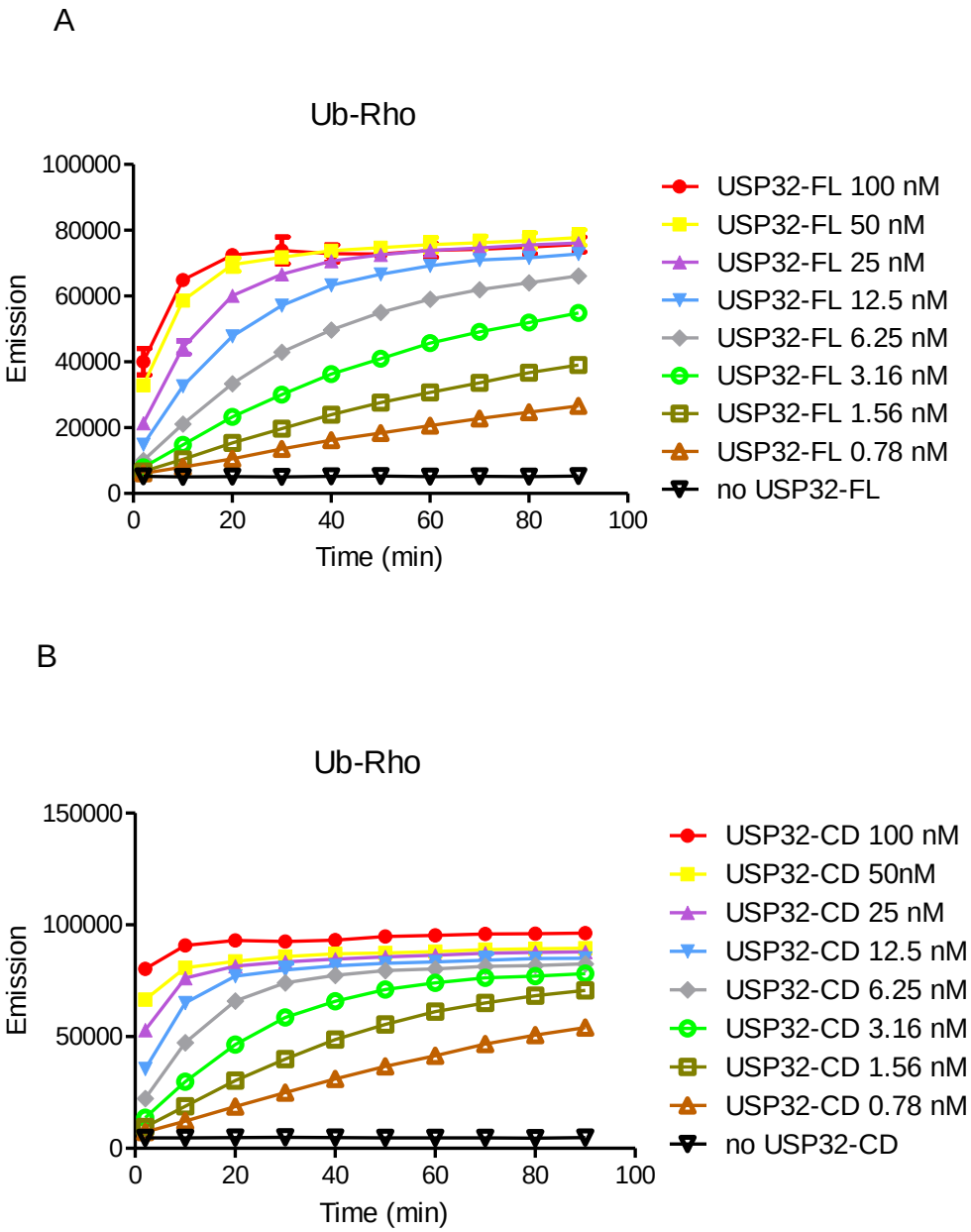



**Figure 3.22.** Deubiquitinating enzyme activity assay with Ub-Rhodamine substrate. **A.** DUB activity assay for USP32-FL protein using Ub-Rho substrate. Gradient concentrations of USP32-FL protein was used for assay in addition to 100 nM Ub-Rho substrate. **B.** DUB activity assay for USP32-CD protein using Ub-Rho substrate. Gradient concentrations of USP32-CD protein was used for assay in addition to 100 nM Ub-Rho substrate. Fluorescent measurements were obtained at every 2 minutes for 90 minutes using PerkinElmer Wallac EnVision 2010 Multilabel Reader. GraphPad Prism 5 and Microsoft office excel software were used for all data analysis.

As a result, USP32-FL and USP32-CD were catalytically active enzymes and could cleave the bond between ubiquitin and rhodamine *in vitro* (Figure 3.23 A and B).

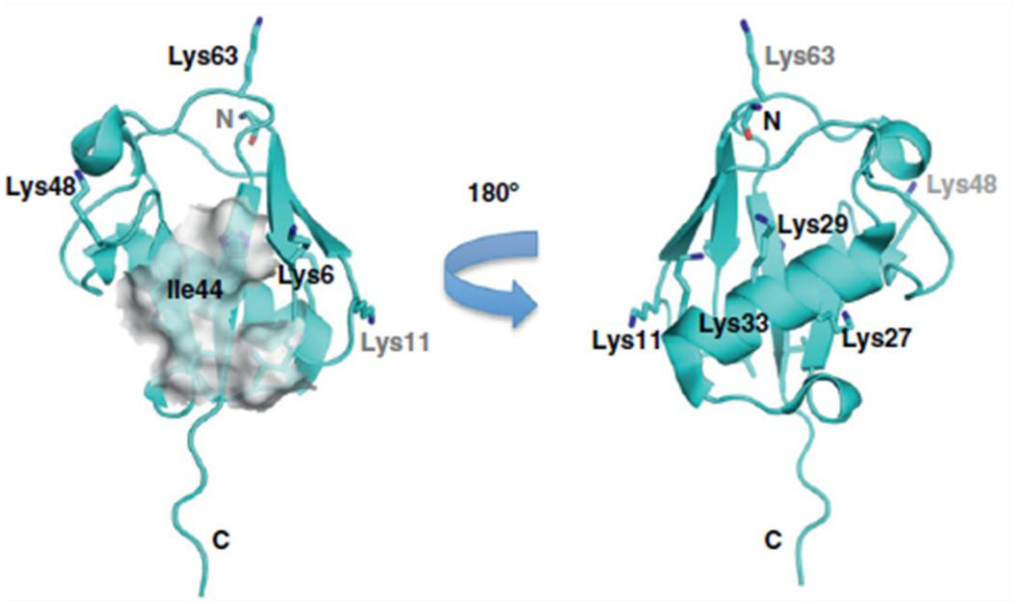
### 3.4. Identification of Ub-linkage specifity of USP32 protein via diubiquitin (Di-Ub) assay

Ubiquitin contains seven lysine residues at 6, 11, 27, 29, 33, 48 and 63 amino acid positions (K6, K11, K27, K29, K33, K48 and K63), all of which form polyubiquitin chains. The types of ubiquitin linkages are important for regulation of different cellular events such as proteosome/lysosomal degradation, membrane trafficking, signal transduction, cell cycle and DNA repair. Although 7 different types of Ub linkages are known, most studies focus on the K48 and K63 linkages [12, 15, 50, 131, 166]. Positions of the lysine residues on the structure of ubiquitin are shown in Figure 3.23A.

Given the important roles of Ub in cells and to have a better understanding of how USP32 functions, in our study, we investigated Ub-linkage specifity of USP32 FL and USP32-CD protein for all seven types of Ub-linkages in addition to the linear-Ub (Met1).

As a result of diubiquitin hydrolysis reactions, we showed that USP32-CD efficiently hydrolyzed all Ub-linkage types except lower efficienciency for linear di-Ub while USP32-FL cleaved all seven types of Ub-linkages except for linear di-Ub (Figure 3.23B)

**A**




**B.**

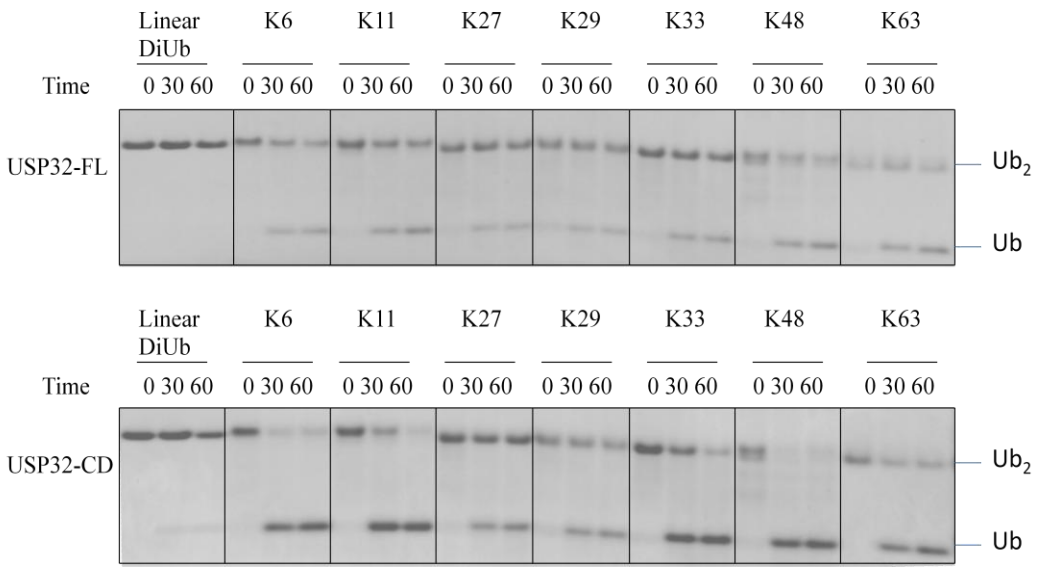



**Figure 3.23.** Di-Ub assay to determine Ub-linkage specifity of USP32 FL and USP32-CD. **A.** NMR structure and all seven lysine residues of ubiquitin (Taken from [167])**. B.** Analysis of Ub-linkage specifity of active USP32-FL and USP32-CD enzymes (37 nM) using all seven lysine-linked di-Ub (5µg) in addition to linear di-Ub (5µg). Samples for each time-course (0, 30 and 60 minutes) were analyzed with coomassie blue stained SDS-PAGE gel.

Interestingly, although USP32 seemed to hydrolyze all seven types of lysine-linked Ubs, there was a clear difference in terms of efficiency. Especially, USP32-FL cleaved K6, K11, K33, K48 and K63 linkage types more rapidly compared to K27 and K29. Similarly, USP32-CD cleaved K6, K11, K33, K48 and K63 linkage types faster than other two linkage types. These results are in agreement with an earlier study on USPs and Ub specificity [63]. USPs investigated in that study hydrolyzed all Ub-linkage types but K27 and K29-linked Ub were slowly hydrolyzed by USPs compared to other linkage types. Interestingly, K6, K11, K48 and K63 residues localize in distinct regions of ubiquitin and in β-sheet or loops. In contrast, K27, K29 and K33 are present in α-helix structures and are more difficult types of linkages for cleavage by USPs [63]. Therefore, hydrolysis efficiency of USP32 may attribute to the linkage types themselves.

### 3.5. Kinetic analysis of USP32-FL and USP32-CD through Ub-FP assay

After establishing that the USP32 is an active and a typical deubiquitinating enzyme, we investigated enzyme kinetics of USP32 using a Ub-Fluorescent polarization (FP) assay. Such kinetic experiments usually favor Ub-AMC substrate for kinetic analysis of DUB enzymes [164, 165, 168, 169]. Unfortunately in that setup, the reporter molecule (AMC) binds to Ub via linear peptide bond and lacks the native isopeptide bond between AMC and Ub. Therefore use of Ub-AMC may not best represent the accurate physiological function of DUBs. To address this issue, new approaches involve synthesis of isopeptide bound Ub-conjugate substrates for DUB enzymes by native chemical ligations. [153, 170, 171].

The basic principle of FP assay is given in Figure 3.25. We used TAMRA-K(Ub)G, fluorescence labeled Ub peptide, as a substrate in this experiment. TAMRA, a fluorophore, is excited at 531 nm and emits the polarized light at 579 nm. When TAMRA connects with a peptide with high molecular weight such as TAMRA-K(Ub)G (indicated as TAMRA-Lys-(Ubl)-X in Figure 3.24), is excited by polarized light, the emitted light results in high polarization (P) value because of the slow

rotational speed of substrate. If TAMRA covalently binds to a small peptide such as TAMRA-KG (shown as TAMRA-Lys-X in Figure 3.25), it is excited by polarized light and emitted light causes low P value due to high rotational speed of the compound. Therefore, DUB activity is demonstrated by proteolysis-depended change of fluorescence polarization in the FP assay [153].

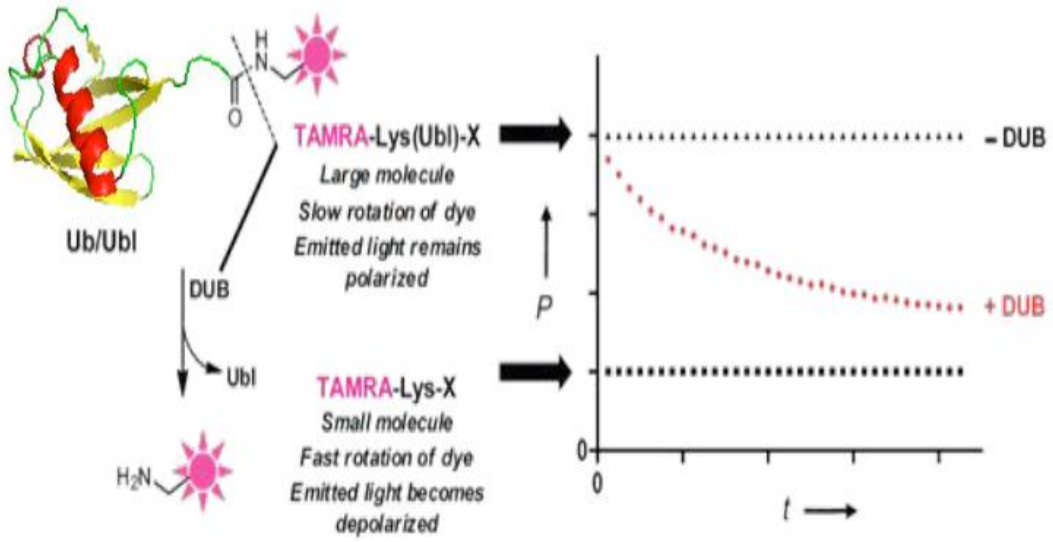
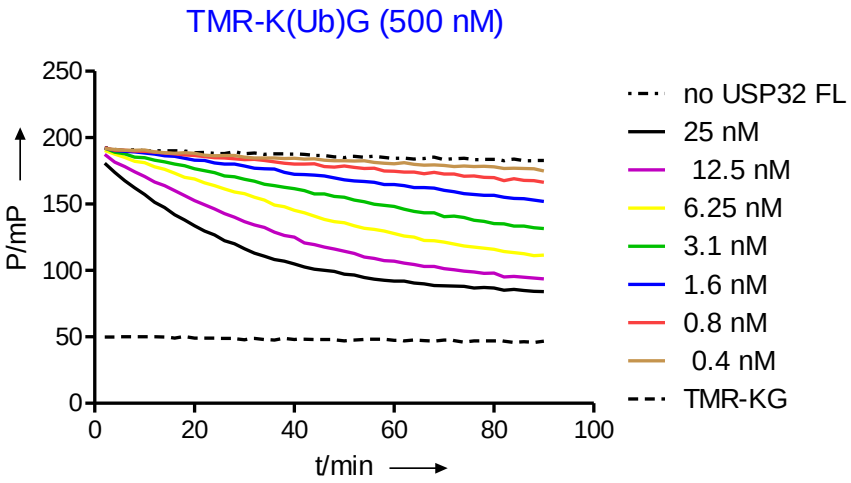



**Figure 3.24.** Basic principle of Ub-fluorescence polarization (Ub-FP assay). Taken from [153].

For the kinetic analysis of USP32-FL and USP32-CD proteins, USP32-FL and -CD proteins were diluted with assay buffer into a concentration gradient (25 nM, 12.5 nM, 6.25 nM, 3.12 nM, 1.56 nM, 0.78 nM, 0.4 nM and 0 nM) to determine the optimum enzyme concentration at which a plot of velocity versus enzyme concentration should be linear. Then, we added TAMRA-K(Ub)G substrate (100 nM) and immediately measured fluorecence using microplate reader. We obtained data every 2 minutes for 1.5 hours (Figure3.25).

A




B

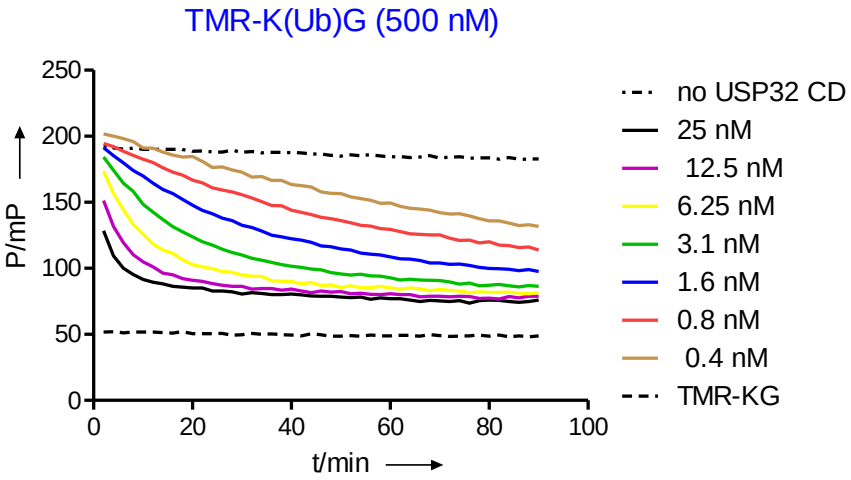



**Figure 3.25.** Ub-FP assay to identify concentration of USP32-FL and USP32-CD for kinetic analysis. **A.** Ub-FP assay to identify convenient concentration of USP32-FL using 8 different protein concentrations with 100 nM TAMRA-K(Ub)G substrate. **B.** Ub-FP assay to identify convenient concentration of USP32-CD using 8 different protein concentrations with 100 nM TAMRA-K(Ub)G substrate. All data were obtained every 2 minutes for 1.5 hours. TAMRA-KG was used as a tracer to obtain lowest polarization value.

After determination of the optimum enzyme concentration, we performed Ub-FP assay for different concentrations of substrate under constant enzyme concentration (10 nM USP32-FL and 1 nM USP32-CD). Diluted enzymes and serial diluted substrate gradients (2000 nM-52 nM) were prepared using the reaction buffer (20 mM Tris-HCl, 100 mM NaCl at pH 7.5 with 5 mM DTT, 0.5 mg/ml BGG, and 10 mg/ml CHAPS). Reaction was started with the addition of the substrate and quantified on PerkinElmer Wallac EnVision2010 multilabel reader with a 531 nm excitation and 579 nm emission filters. Non binding surface flat bottom low flange black 384 well plate were used for the assay. From the obtained polarization value (P), the amount of processed substrate (Pt) was calculated with the following equation 3.1,

$$S = S0 - S0\ x\ \left(\frac{Pt - Pmin}{Pmax - Pmin}\right)$$

S= the amount of processed substrate

S0= the amount of substrate added to the reaction

Pt= polarization measured from USP32-FL or CD+Substrate reaction in the time point

Pmax= polarization measured from only substrate reaction in the time point

Pmin= polarization measured from only control peptide without USP32-FL or -CD and substrate reaction in the time point.

After calculating the amount of converted substrate, we characterized properties of enzyme kinetics. For this aim, Km, kcat and Vmax value were firstly analyzed using **Michaelis-Menten equation**. To calculate Km, kcat and Vmax, we needed to determine reaction velocity for each different substrate concentration, termed as

initial velocity ($V_0$). $V_0$ was calculated from slope of plot of amount of converted substrate versus time using following equation 3.2;

$$V0 = \frac{\Delta S}{\Delta t}$$

$V_0$= initial rate of converted substrate or product formation per unit time

S= the amount of converted substrate

t= unit time

After calculating $V_0$, we obtained graph of $V_0$ versus concentration of substrate shown in Figure 3.26 by using GraphPad Prism5 software and nonlinear regression analysis with Michaelis-Menten equation.

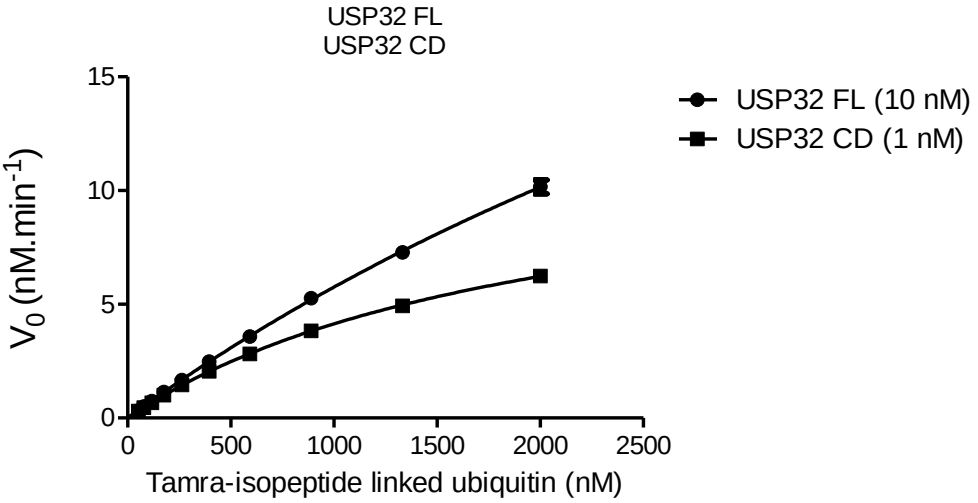



**Figure 3.26.** The Michaelis-Menten curves for USP32-FL and USP32-CD by using plot of initial velocity, $V_0$, versus different substrate (Tamra-Isopeptide linked ubiquitin) concentrations (0-2000 nM) at constant enzyme concentrations (10nM USP32-FL and 1 nM USP32-CD). All data analysis was performed using Microsoft Excel and GraphPad Prism5 softwares.

Vmax, Km (**Michaelis constant)**, and kcat called **turnover number** were calculated by following equation 3.3 and 3.4, respectively;

$$V0 = Vmax\frac{[S]}{Km + [S]}$$

$$kcat = \frac{Vmax}{[Et]}$$

The kinetic parameters for USP32-FL and USP32-CD enzymes are summarized in Table 3.1.

**Table 3.1.** Overview of kinetics parameters for USP32-FL and USP32-CD

| Enzyme | FP Substrate | kcat ($s^{-1}$) | Km (µM) | kcat/Km ($M^{-1}.s^{-1}$) |
|---|---|---|---|---|
| **USP32 FL** | TMR-K(Ub)G | 0.07165±0.00905 | 6.471±0.999 | 11.07 x $10^3$ |
| **USP32 CD** | TMR-K(Ub)G | 0.2095±0.007 | 2.037±0.102 | 10.2 x $10^4$ |

Taken together, our data showed that USP32-FL seemed to have higher affinity to its substrate (Ub) than USP32-CD when their $K_m$ values are compared (6.471>2.037) because $K_m$ is the concentration of substrate at which $V_0$ corresponding to half of $V_{max}$ and low $K_m$ value means that a small amount of substrate concentration is enough to realize enzymatic reaction at half of maximum speed ($V_{max}$). Therefore, low $K_m$ value indicates high affinity of enzyme for the substrate.

On the other hand, $k_{cat}$ value measures catalytic production of reaction product at unit time per enzyme molecule and indicates catalytic turnover. Therefore, USP32-FL displayed about 3-fold lower catalytic turnover efficiency than USP32-CD. In addition, $k_{cat}/K_m$ rations, a constant for substrate specificity, for USP32-FL and USP32-CD demonstrated that USP32-FL exhibited approximately 10-fold lower substrate specificity than USP32-CD (Table 3.1).

The fact that USP32-FL had lower affinity for substrate suggested a possible role for the $Ca^{+2}$ binding domain and DUSP (domain present in ubiquitin specific proteases) domain of USP32. We may speculate that $Ca^{+2}$ binding domain may be causing structural rearrangement of ubiquitin binding site and active site of USP32 and this may affect the affinity/activity of USP32. Moreover, DUSP domain may also be associated with protein/protein interactions and may cause intermolecular modulation of USP32. This results in modulation of USP32 activity and substrate affinity.

# CHAPTER IV

## CONCLUSION

Gene amplification is a common mechanism of oncogene activation in cancer. Previous studies identified 17q23 as a frequent site of gene amplification in breast cancer. Amplification and overexpression of oncogene candidate genes in this region could be important for breast cancer. One of the oncogene candidate genes on this region is *USP32* (Ubiqiuitin specific protease 32). Our laboratory showed overexpression of USP32 to cause altered cell growth, cell motility and migration in breast cancer cells.

USP32 belongs to ubiquitin specific protease (USP) subfamily of DUBs based on domain structure. Results of amino acid sequence alignments between USP32 and 3 other active DUBs showed presence of common DUSP (*domain* present in ubiquitin-specific proteases), Cys, and His domains found in DUBs.

Deubiquitinating enzymes are important regulators in many cellular events including protesomal/lysosomal degradation, signal transduction, membrane trafficking, DNA repair, gene transcription. They also play crucial roles in the pathogenesis of several diseases such as, cancer and neurogenerative disorders.

To date, about 90 DUBs are detected in humans as active enzymes which belong to different DUB subfamilies. Some of DUBs have been already identified as active enzymes but some others like USP32 remains as predicted DUB enzymes.

In this study, we determined USP32 as a novel active deubiquitinating enzyme both *in vivo* and *in vitro*. We also investigated substrate specificity of full length (USP32-FL) and catalytic domain (USP32-CD) of USP32. For this aim, we used 7 Ub substrates that represented different types of Ub-linkage as well as linear diubiquitin. As a result, USP32-FL and USP32-CD cleaved K6, K11, K33, K48 and

K63 linkage types more rapidly compared to K27 and K29. This result may be associated with the positions of lysine residues. K6, K11, K48 and K63 residues localize in distinct regions of ubiquitin and in β-sheet or loops. In contrast, K27, K29 and K33 are present in an α-helix structure and are predicted to be more difficult types of linkages to be cleaved by USPs. Especially, K27 and K29 localize in the middle of ubiquitin protein and they may be more difficult than K33 type of diubiquitin to be cleaved. K29-, K33-, linear- (called as Met1-linked) and K63-linkages form extended conformations while K6-, K11-, K27- and K48- linked chains lead to closed conformation in the ubiquitin chain structure. The fact that USP32 can cleave all, with different efficiencies, substrate specificity of USP32 does not seem to be completely dependent on the ubiquitin chain structure conformation. USP32 can cleave mixed or branched linkage types.

Types of Ub linkages are have been associated with specific cellular events. For example, most of K11 linked ubiquitin chains are produced during mitosis and are critical for cell cycle control [172]. Additionally, K11-linked ubiquitin chains are synthesized when endoplasmic reticulum (ER) stress is induced and therefore, K11 linked ubiquitin chains play a role in endoplasmic reticulum associated degradation [25].

K48-linked ubiquitin chains have a role in proteosomal degradation of target proteins. For instance, K48-linked polyubiquitylation of IκBα leads to proteosomal degradation of IκBα and allows to NF-κB translocation from cytoplasma to nucleus [173, 174]. K48 linked ubiquitin chains together with K11, linear and K63 linked ubiquitin chains are also associated with the regulation TNF induced NF-κB activation by polyubiquitylation of RIP1 [175]. K63-linked ubiquitin chains in the NF-κB signaling pathway and DNA repair are associated with the nondegradative ubiquitin signal[176]. K6-linked ubiquitin chains are associated with the formation of conjugated ubiquitin by BRCA1: BARD1 complex and this suggested that K6-linked may be related with DNA repair mechanism [177]. Hence, due to the ability to cleave all different types of Ub linkages, it is difficult to propose a cellular

function for USP32 based on its DUB specifity. Other functional assays will be needed to delineate a more specific function for USP32.

We also investigated kinetic properties of USP32 enzyme using full length and catalytic domain constructs of USP32 (USP32-FL and USP32-CD). We detected that USP32-FL had higher affinity to its substrate (Ub) than USP32-CD. This finding may suggest the significance of the tertiary structure of USP32. There are several reasons to explain the different catalytic turnover and substrate (Ub) binding efficiencies of USP32-FL and USP32-CD. USP32-FL harbors $Ca^{+2}$ binding domain and DUSP (Domain present in ubiquitin specific protease) domain but USP32-CD does not contain these domains. USP32 is the only known DUB with a $Ca^{+2}$ binding domain and $Ca^{+2}$ binding domain is associated with conformational change of protein upon $Ca^{+2}$ binding. Therefore, the conformational change of USP32 due to this domain may affect substrate binding and its catalytic efficiency. Moreover, the function of DUSP domain is unknown. This domain may be associated with protein/protein interaction. The presence of an interacting partner may also affect its substrate affinity and catalytic efficiency.

Taken together, further investigations will be needed to understand the effect of these domains on the activity of USP32. Identification of USP32 interacting partners and/or substrate will give us a better understanding of its functions in cells, as well. More detailed follow up studies will give us a better idea about how USP32 may be important in normal and in cancer cells. Moreover, further characterization assays to reveal the functions of $Ca^{+2}$ binding will be needed. Understanding function and cellular interaction of USP32 will help us reveal its importance in cellular events.

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## APPENDIX A

### Primers

**Table A.1.**USP32 sequencing primers (5'-->3')

| **Primer Name** | **Primer Sequence (5'-->3')** |
|---|---|
| CDS1F | TCTCGATGGCTTCTATCTGGA |
| CDS1R | CACCTGGAAAAGGAGGTTCA |
| CDS2F | GACACCACAAAGATGGGTCA |
| CDS2R | TCAATAGCCCCTGGTTTCTG |
| CDS3F | CTGAAGCCTCAGAAACTGCT |
| CDS3R | CTCTTTAATGCGCAGCCTTT |
| CDS4F | CCCCGCTATCTTCTCTTCCT |
| CDS4R | TTTAGCCATATGCCCCTTCA |
| CDS5F | TGAACTCAACAGGACAAATCC |
| CDS5R | CCACAGAGATCACTCAGCTGTT |
| CDS6F | ATGGGTTTCAGCAACAGGAC |
| CDS6R | TGGCGAAGAGGAGAAATCTG |
| CDS7F | CCTGTCCCTGTGTCTCCAAT |
| CDS7R | AAGCTCTGTCTTCCCCACAA |
| CDS8F | GATGCGGTTTGGATTCAAGT |
| CDS8R | AGAGCCGGGTCTCTTGGTA |
| CDS9F | CAAAGAAGCTGGATCTCTGGA |
| CDS9R | GGTCCTGAGGAGTGACCAAC |
| CDS10F | CTGCCAGCATAAACCACTCA |
| CDS10R | GAATGGCACGAAATTGCATA |
| pGEX4T2F | GTATATAGCATGGCCTTTGCAG |
| pGEX4T2R | GAGCTGCATGTGTCAGAGG |
| GW1 | GTTGCAACAAATTGATGAGCAATGC |
| GW2 | GTTGCAAGAAATTGAGCAATTA |

**Table A.2.** USP32 cloning primers (5'-->3')

| **Primer Name** | **Primer Sequence (5'-->3')** |
|---|---|
| USP32-I FW | ACGCGTCGACTAATAACAACCAGTGTTTGCT |
| USP32-I RV | ATAAGAATGCGGCCGCTTAGAGGCTGGGCGATTCTT |
| USP32-II FW | ACGCGTCGACTAATAACAACCAGTGTTTGCT |
| USP32-II RV | ATAAGAATGCGGCCGCTTACTGTAACACACAGTACTTT |
| USP32-III FW | ACGCGTCGACTCCTGTGTCTCCAATTTCAGCT |
| USP32-III RV | ATAAGAATGCGGCCGCTTACTGTAACACACAGTACTTT |
| USP32-FL FW | CAGGGACCCGGTGGTGCCAAGGAGTCACGGATC |
| USP32-FL RV | CGAGGAGAAGCCCGGTTACTGTAACACACAGTACTTTT TGTAATCAGACTC |
| USP32-CD FW | CAGGGACCCGGTAGTAAAATAGATAGACACAAGGT TCCCACAG |
| USP32-CD RV | CGAGGAGAAGCCCGGTTACTGTAACACACAGTACTTTT TGTAATCAGACTC |

## APPENDIX B

### Vectors

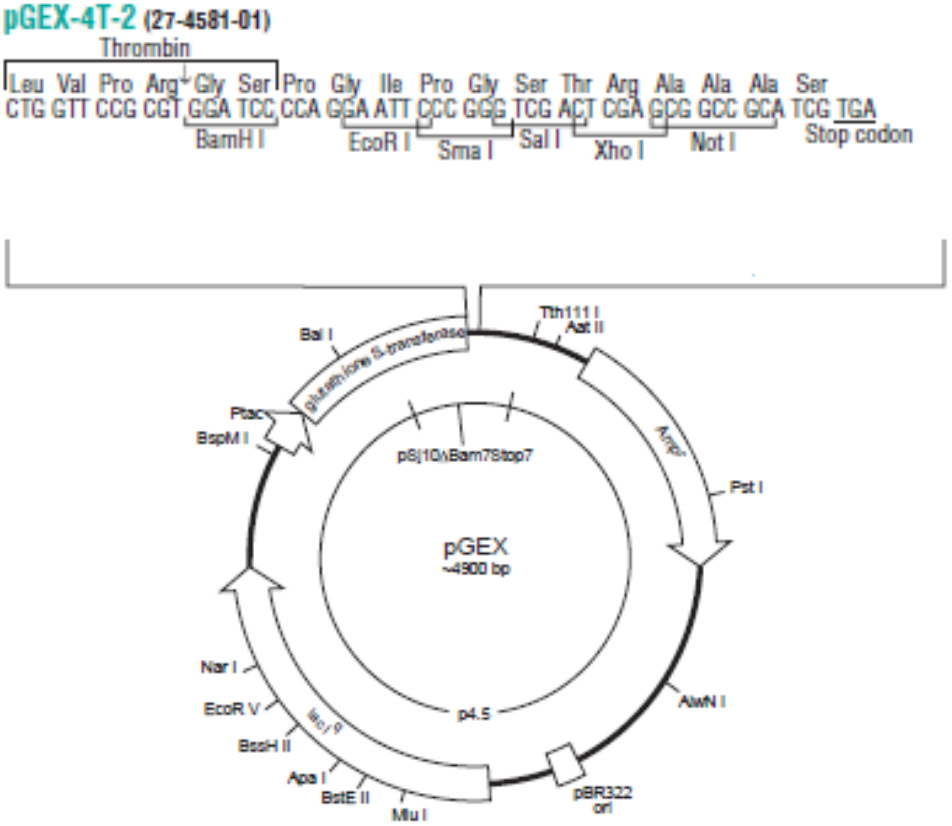



**Figure B.1.** The map and multiple cloning site of pGEX-4T-2 vector (Amersham)

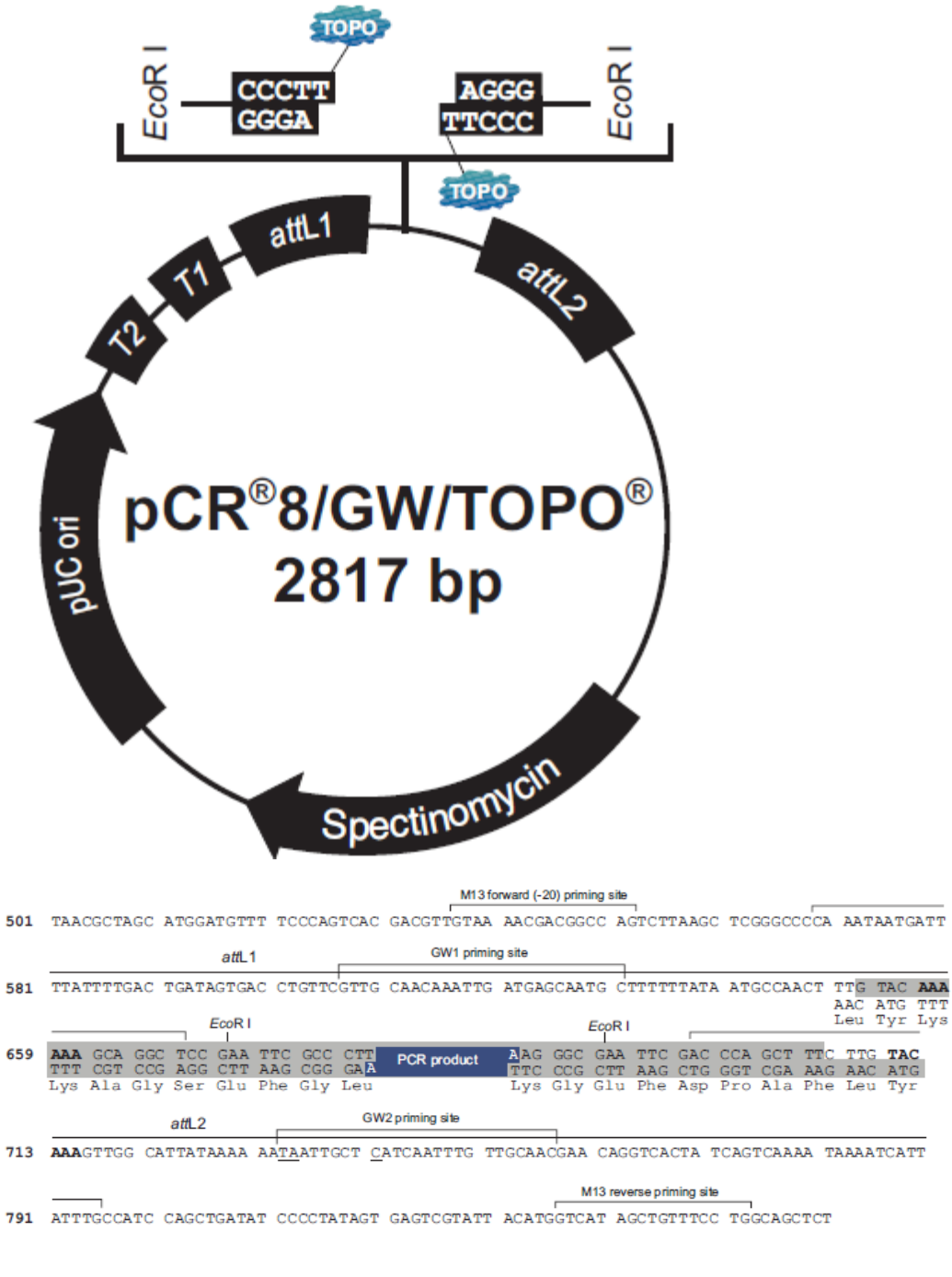
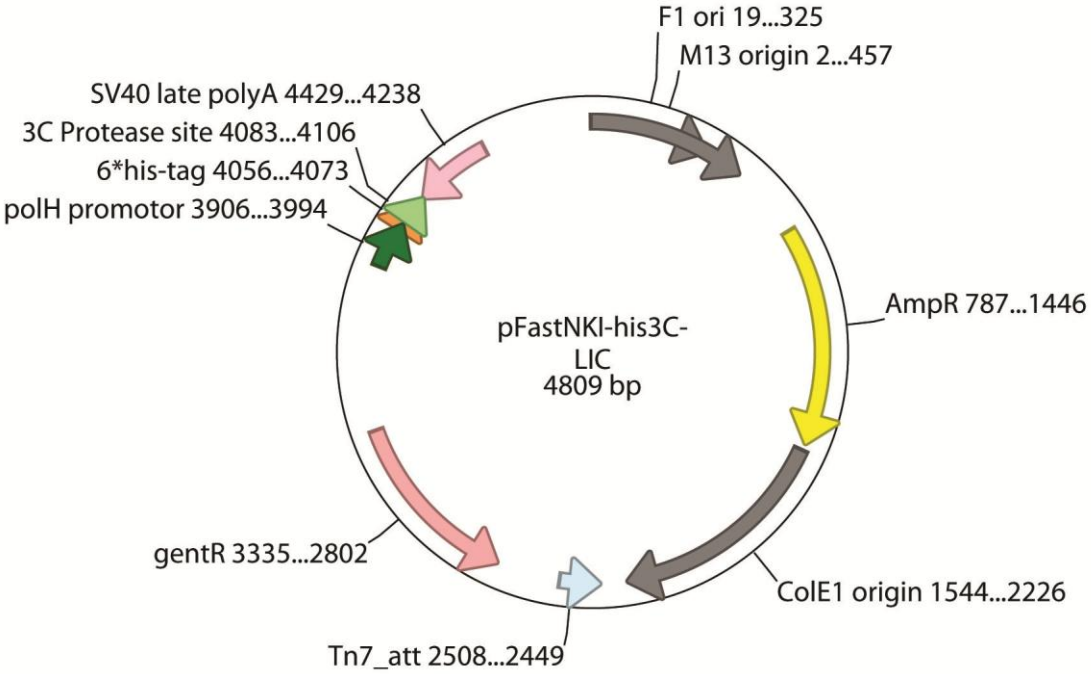



**Figure B.2. The map and multiple cloning site of** PCR 8/GW/TOPO cloning vector (Invitrogen).




```
                  4048 NcoI                                                 4108 KpnI
                  |                                                         |
4035 TCTCGGTCCGAAACCATGGCACATCACCACCACCATCACTCCGCGGCTCTTGAGGTGCTCTTTCAGGGACCCGGGTACCAAGAACAAAAACTCATCTCAG 4134
                       >>>>>>>>>>>>>>>>>>          >>>>>>>>>>>>>>>>>>>>>>>>>
                       6*his-tag                  3C Protease site

                                          4170 HindIII
                  4148 KpnI       4164 XhoI
                  |               |     |
4135 AAGAGGATCTGTTGGTACCCGGGCTTCTCCTCGAGAAGCTTG 4176
```

**Figure B.3.** The map and multiple cloning site of pFastNKI-his3C-LIC cloning vector.

## APPENDIX C

## Buffers, Solutions and Growth Media

### TBE Buffer (5X)/L

| | |
|---|---|
| Tris base | 54 g |
| Boric Acid | 27.59 g |
| 0.5 M EDTA (Ph 8.0) | 20ml |

Stored at room temperature.

### TAE Buffer (5X)/L

| | |
|---|---|
| Tris base | 24.2 g |
| Acetic Acid | 5.71 ml |
| 0.5 M EDTA (Ph 8.0) | 10ml |

Stored at room temperature.

### LB-Media (Luria-Bertani Media)

| | |
|---|---|
| Tryptone | 10 g |
| Yeast Extract | 5 g |
| NaCl | 10 g |

Add dd$H_2O$ to 1 liter. The pH was adjusted to 7 with using NaOH (5 N). The media was sterilized by autoclaving for 20 minutes at 15 Psi.

**LB-Agar**

| | |
|---|---|
| Bacto Agar | 15 g |
| Tryptone | 10 g |
| Yeast Extract | 5 g |
| NaCl | 10 g |

Add dd$H_2O$ to 1 liter. The pH was adjusted to 7 with using NaOH (5 N). The media was sterilized by autoclaving for 20 minutes at 15 Psi.

**S.O.C Media**

| | |
|---|---|
| Tryptone | 20 g |
| Yeast Extract | 5 g |
| NaCl | 0.5 g |
| 250 mM KCl | 10 ml |

The volume was adjusted to 1 liter. The pH was adjusted to 7, using NaOH (5 N). The media was sterilized by autoclaving for 20 minutes at 15 Psi. 20 ml of sterile 1M solution of glucose was added to the cooled media. Just before use, 5 ml of a sterile solution of MgCl2 (2M) was added to the media.

**2X YTA media**

| | |
|---|---|
| Tryptone | 16 g |
| Yeast Extract | 10 g |
| NaCl | 5 g |

Add dd$H_2O$ to 1 liter. The pH was adjusted to 7 with using NaOH (5 N). The media was sterilized by autoclaving for 20 minutes at 15 Psi.

## APPENDIX D

## Markers

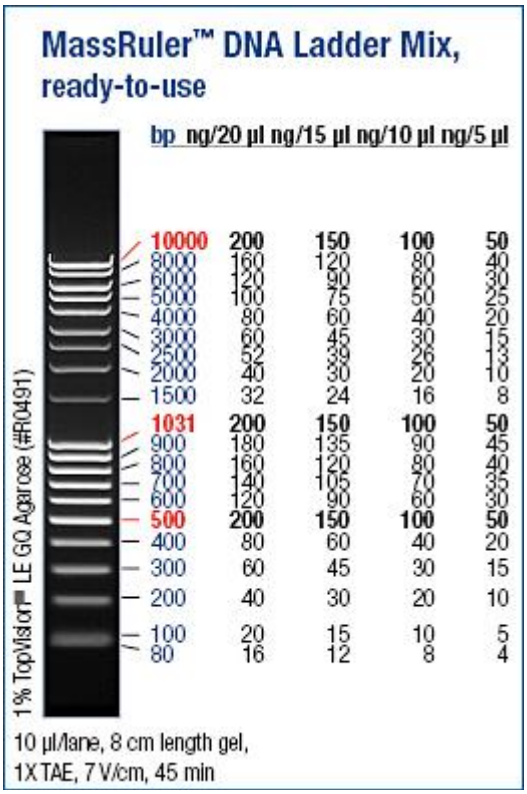



**Figure D.1.** Mass Ruler DNA Ladder Mix (Fermentas, catalog no: R0491).

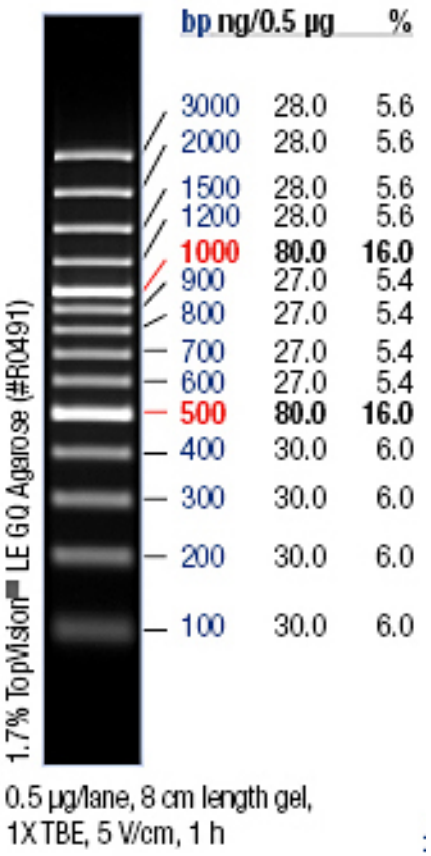



**Figure D.2.** GeneRuler 100bp DNA Ladder Mix (Fermentas, catalog no: SM0321/2/3).

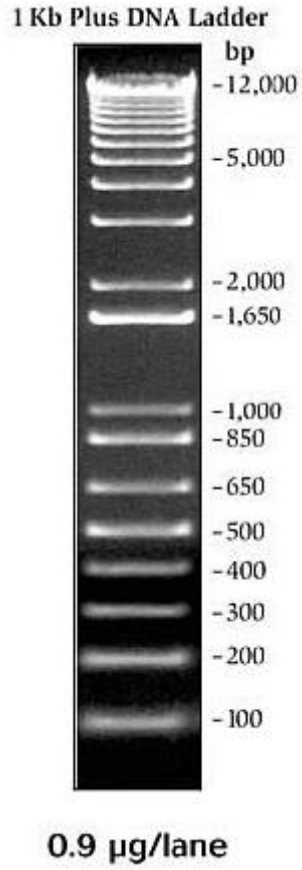



**Figure D.3.** 1 Kb plus DNA ladder (Invitrogen catalog no: 10787-018).

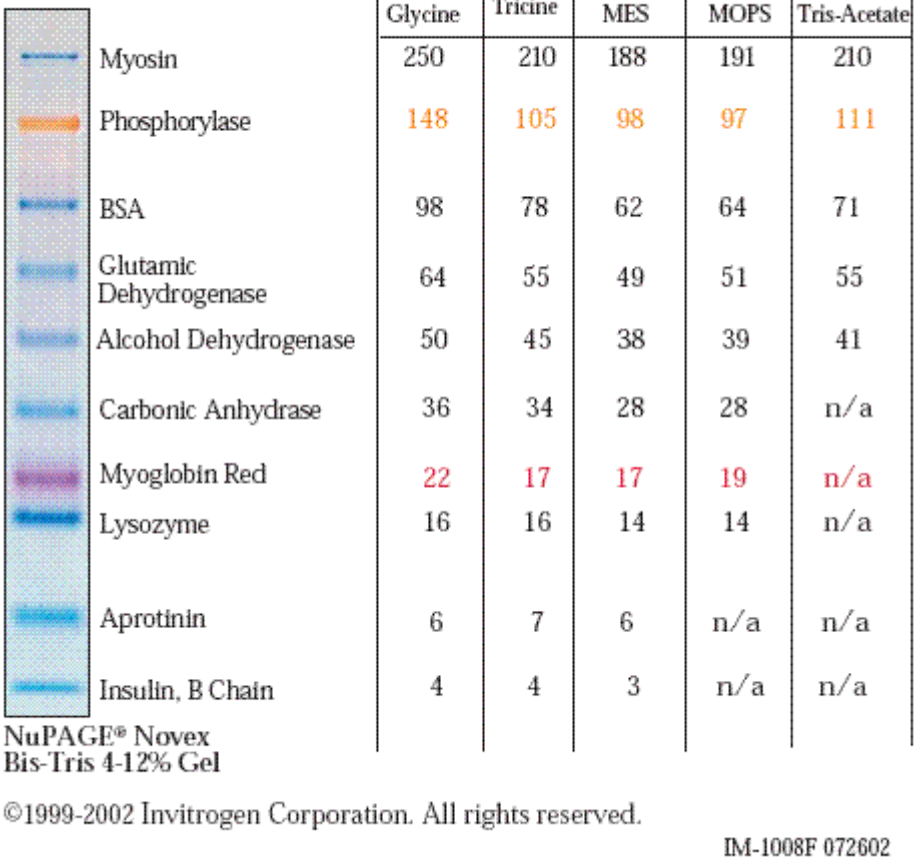



**Figure D.4.** SeeBlue® Plus2 Pre-Stained Standard (Invitrogen, catalog no: LC5925)

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1. Akhavantabasi S, **Sapmaz A**, Tuna S, Erson-Bensan AE. miR-125b targets ARID3B in breast cancer cells. Cell Struct Funct. 2012;37(1):27-38.
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**Abstracts and Presentations:**

1. Akman Tuncer HB, **Sapmaz A**, Akhavantabasi S, Erson AE. Characterization of a ubiquitin specific protease in the 17q23 amplicon. The European Association for Cancer Research 21st meeting, Oslo, Norway, June 26-29 2010,
2. **Sapmaz A**., Akhavantabasi S., Korkmaz M., Erson A.E., Investigation of the effect of different boron concentration on breast cancer cell proliferation. XI.

National Medical Biology and Genetics Congress, 28-31 October 2009. Nevşehir, Turkey.

3. **Sapmaz A**, Erson AE. Functional Characterization of an Oncogene Candidate, USP32, in Breast Cancer. Int. Symposium on Biotechnology, Ankara, Turkey, Sept. 27-30, 2009
4. **Sapmaz A**, Akhavantabasi S, Petty EM, Erson AE. Characterization of an Oncogene Candidate, USP32, on 17q23, European Human Genetics Conference, Austria Center Vienna, May 23 - 26, 2009
5. **Sapmaz A**, Yuce R, Erson AE, Identification of the function and regulation mechanism of USP32, EMBO YOUNG SCIENTISTS FORUM, Istanbul, Turkey, Feb 20-22, 2008.
6. Sapmaz A., Özen Karataylı S.C., Dağlı U., Kılıç Z.M., Törüner M., Çelik Y., Özkan M., Soykan I., Çetinkaya H., Ülker A., Özden A., and Bozdayı A.M.**. Correlation between** Multidrug Resistance 1 (Mdr1) Single Nucleotide Polymorphisms and Inflammatory Bowel Disease. 23th National Gastroenterology Congress, 4-7 November 2006, Istanbul, Turkey.

**Awards and Honors:**

- Full PhD scholarship from Higher Education Council (2005-present)
- **The Scientific and Technological Research Council of Turkey (TUBITAK)- National Scholarship Programme for PhD Students (2006-2010) (**a scholarship programme for students pursuing a PhD in a university in Turkey **(about 700-800 PhD students supported per year)**.
- Publication Award from Middle East Technical University for Mammalian Genome. 2010 Aug; 21(7-8): 388-397
- Publication Award from TUBITAK for Mammalian Genome. 2010 Aug; 21(7-8): 388-397
- Travel Grant for European Human Genetics Conference, Austria Centre Vienna, May 23 - 26, 2009.

- Awards for second prize (23th National Gastroenterology Congress, 4-7 November 2006, Istanbul, Turkey)
- Highest cumulative graduate student award (2003-2004) Biotechnology Institute, Ankara University.
-

**Laboratory Experience:**

**Cell Biology and Genetics**

- Designing of Cloning experiments (Ligation dependent and independent cloning)
- Bacterial transformation and expression studies
- Mammalian cell culture and transfections
- Overexpression and silencing via shRNA

**Expression Analysis**

- Semiquantitative RT-PCR
- Real Time PCR
- Western Blotting

**Functional analysis**

- *In vitro* and *In vivo* deubiquitination assay
- Cell characterization assays (cell proliferation, migration, and motility etc.)
- Dual-luciferase assay
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**Protein expression and purification facility**

- Protein expression in *E.coli,* Insect cells and mammalian cells
- GST-tag and His-Tag protein purification

- Gel filtration
- Anion-Cation exchange purification