# Studies on Substrate Recognition Mechanism and Physiological Function of Rpn10, A Subunit of 26S Proteasome

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# Studies on Substrate Recognition Mechanism and Physiological Function of Rpn10, A Subunit of 26S Proteasome

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# Abstract

In all kinds of cells, proteins are constantly synthesized while unnecessary proteins are simultaneously degraded and eliminated by various kinds of proteases. Protein degradation is important to eliminate damaged proteins, produce a pool of amino acids in response to starvation, and regulate cellular functions, immune response, and so on. If protein degradation system is dysregulated, damaged, oxidized or degenerated proteins accumulate inside the cells, which would be toxic for organisms and could cause various diseases. Thus, protein homeostasis is strictly regulated in a temporal and spatial manner to maintain cell survival, growth, and functions.

The ubiquitin-proteasome system plays a central role in degradation of intracellular proteins in eukaryotic cells. The 26S proteasome is a protease complex consisted of approximately 30 subunit proteins. Recognition of polyubiquitinated proteins by the 26S proteasome is considered as a key step in the selective degradation. The Rpn10 subunit of the 26S proteasome can bind to polyubiquitylated proteins via its ubiquitin-interacting motifs and plays a role in the interaction of the 26S proteasome with the substrates. Interestingly, it has been reported that there is structural diversity in Rpn10 subunits. Five distinct Rpn10 isoforms, Rpn10a, Rpn10b, Rpn10c, Rpn10d, and Rpn10e, are generated from a single gene by alternative splicing of mRNA in mouse. Since each Rpn10 isoform contains a unique C-terminal stretch, it is speculated that different Rpn10 isoform can interact with different proteins. However, it has been unclear whether such alternative splicing products are observed in other species than mice. In addition, it has been undetermined that each Rpn10 isoform plays isoform-specific function.

To elucidate the interspecies conservation of Rpn10 isoforms, I searched for Rpn10 cDNAs in databases and in my original PCR products from human, mouse, and

rat, in the first chapter. I also clarified the genomic organization of Rpn10 gene in lower vertebrates and provided evidence for the competent generation of distinct forms of Rpn10 by alternative splicing of mRNA through evolution. In the second chapter, I elucidated a specific function of one of the alternative-splicing products of *Xenopus* Rpn10, named Xrpn10c. I revealed that Xrpn10c functions as a specific receptor for Scythe that has been reported as an anti-apoptotic protein. My study showed that Xrpn10c has a Scythe-binding site at its C-terminal region and the forced expression of a Scythe mutant protein lacking Xrpn10c-binding domains in *Xenopus* embryos induces embryonic death, while the wild-type Scythe did not show any abnormality. These results from my studies indicate that Rpn10 family are largely conserved among vertebrates and may play an important role in the regulation of animal development probably via temporally controlled protein degradation.

# Abbreviations

| | |
|---|---|
| ATP | adenosine triphosphate |
| BAG | BCL2-associated athanogene |
| BAT | human leukocyte antigen B (HLA-B)-associated transcript |
| CHIP | C-terminus of Hsc70-interacting protein |
| CS | conserved sequence |
| DDBJ | DNA Data Bank of Japan |
| DNA | deoxyribonucleic acid |
| EMBL | European Molecular Biology Laboratory |
| ER | endoplasmic reticulum |
| ES | embryonic stem |
| EST | expressed sequence tag |
| FBS | fetal bovine serum |
| FL | full-length |
| GFP | green fluorescent protein |
| GST | glutathione S-transferase |
| HECT | homologous to the E6AP carboxyl terminus |
| HR23 | homologue of Rad23 |
| IFN | interferon |
| IP | immunoprecipitation |
| KI | knock-in |
| KO | knockout |
| LRR | leucine-rich-repeat |
| PAGE | polyacrylamide gel electrophoresis |
| PCR | polymerase chain reaction |

| | |
|---|---|
| PLIC | protein linking integrin-associated protein to cytoskeleton |
| PUbS | polyubiquitin site |
| RBR | ring between ring fingers |
| RING | really interesting new gene |
| RT | reverse transcription |
| SDS | sodium dodecyl sulfate |
| UBA | ubiquitin-associated |
| UBL | ubiquitin-like |
| UIM | uiquitin-interacting motif |
| VWA | von Willebrand factor A |

# General Introduction

## Ubiquitin-Proteasome System

Proteins are synthesized to maintain cell functions while damaged, degenerated, oxidized, or unnecessary proteins are simultaneously degraded in a timely manner. In other words, there is a balance between protein synthesis and protein degradation, and not only protein synthesis but protein degradation is indispensable for cells. Therefore, it is important to study how degradation of intracellular proteins is strictly regulated to maintain precise cellular functions.

The ubiquitin-proteasome system plays a crucial role in a temporal and spatial degradation of intracellular proteins in eukaryotic cells (Fig. 1) (Coux et al., 1996). Ubiquitin is a small protein which consists of 76 amino acid residues. In the ubiquitin-proteasome system, ubiquitin covalently attaches to lysine residue(s) of target protein through its C-terminal glycine, changing the stability, function, or localization of the target protein (Haglund and Dikic, 2005; Hershko and Ciechanover, 1992). Ubiquitin also attaches to lysine residues of another ubiquitin

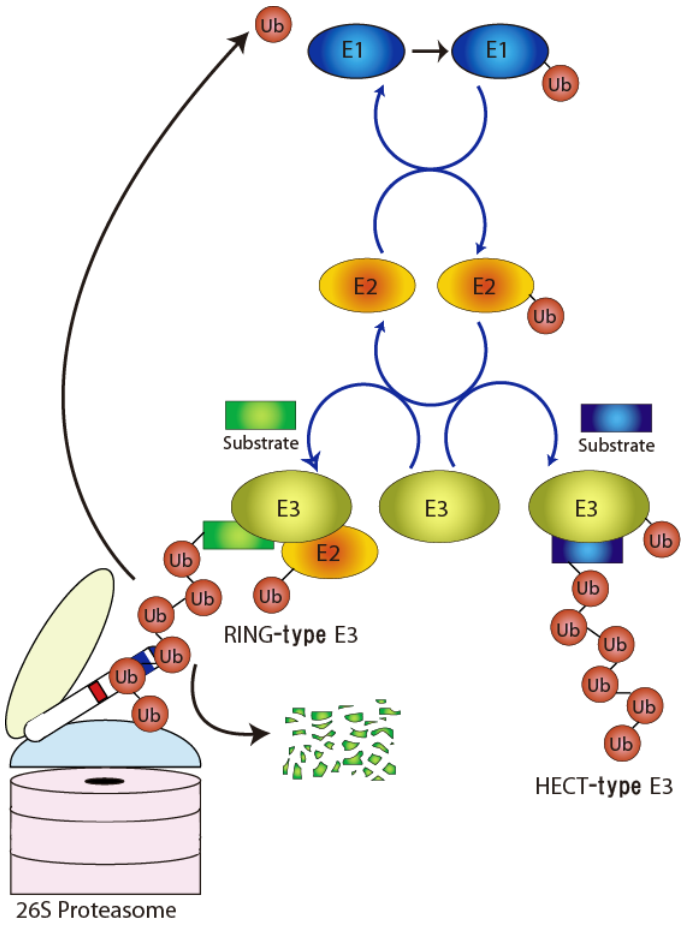



**Fig. 1. Ubiquitin-proteasome system**

to form polyubiquitin chains. This post-translational modification of proteins is called ubiquitination or ubiquitylation. Ubiquitination is mediated by ubiquitin-activating enzymes (E1), ubiquitin-conjugating enzymes (E2), and ubiquitin ligases (E3). First, E1 catalyzes the ATP-dependent activation of ubiquitin and formation of a thioester bond between ubiquitin C-terminus and the catalytic cysteine of the E1. Ubiquitin is then transferred to a catalytic cysteine of an E2 and through an E3 ligase to substrate proteins. Then, the 26S proteasome recognizes polyubiquitinated proteins as substrates and mediates selective protein degradation in an ATP-dependent manner (Hershko and Ciechanover, 1998). Collectively, the ubiquitin-proteasome system-mediated protein degradation comprises the two step reactions – the first step is the polyubiquitination of substrate proteins and the second step is the recognition and degradation of polyubiquitinated substrate proteins by the 26S proteasome.

The ubiquitin-proteasome system is highly conserved in eukaryotes. Many proteins implicated in various cellular functions have been reported to be broken down through this system. For instances, the ubiquitin-proteasome system is involved in control of cell cycle (Kawahara and Yokosawa, 1992; Yew, 2001), cell signaling (Lai, 2002; Lohi and Lehto, 2001), stress response (Parag et al., 1987), endoplasmic reticulum (ER)-mediated protein quality control (Sommer and Wolf, 1997), apoptosis (Jesenberger and Jentsch, 2002; Tanaka and Kawahara, 2000), and immune response (Ben-Neriah, 2002). In addition, the ubiquitin-proteasome system is considered to contribute to various human diseases such as cancer, inflammation, and neurodegenerative diseases (Gong et al., 2016; Paul, 2008; Schwartz and Ciechanover, 1999; Shen et al., 2013; Wang and Maldonado, 2006).

As mentioned above, the ubiquitin-proteasome system recognizes various types of functional proteins as substrates. However, it has been reported that there are only two subtypes of E1 enzymes and fewer than 40 subtypes of E2 enzymes while E3 consists of a large family of more than 600 proteins, suggesting that E3 ligases play an important role in the selection and recognition of substrate proteins to be degraded by the 26S proteasome (Deshaies and Joazeiro, 2009). The E3 ligase family is largely classified into two subfamily, HECT (homologous to the E6AP carboxyl terminus)-type E3 ligases (Zachariae and Nasmyth, 1999) and RING (really interesting new gene)-type E3 ligases (Jackson et al., 2000; Saurin et al., 1996). HECT-type E3 ligases and RING-type E3 ligases have distinct mechanisms of ubiquitin transfer to their substrate proteins. HECT-type E3 ligases catalyze ubiquitin transfer to the substrate protein through a two-step reactions; ubiquitin is first transferred to a catalytic cysteine of an E3 and then from the E3 to the substrate. On the other hand, RING-type E3 ligases transfer ubiquitin directly to substrate proteins. In this respect, RING-type E3 ligases are more like scaffold proteins than enzymes. Some RING-type E3 ligases form a protein complex composed by multiple subunits with F-box proteins (e.g. Cullin and Skp-1). There are also small E3 ligase subfamilies called U-Box-type E3 ligases (Aravind and Koonin, 2000; Hatakeyama et al., 2001), which mediates a direct transfer of ubiquitin to the substrate, and RBR (ring between ring fingers)-type E3 ligases (Dove et al., 2016; Spratt et al., 2014) which catalyzes ubiquitin transfer through two-step reactions. Taken together, the heterogeneity of E3 ligases contributes to substrate specificity and dynamic regulation of intracellular protein degradation.

## Structure of 26S Proteasome

The 26S proteasome is a protease complex consisted of approximately 30 subunit proteins (Voges et al., 1999). The 26S proteasome is composed of two sub-complexes called the 20S proteolytic core complex (i.e. 20S proteasome) and the 19S regulatory complex attached to the both ends of the 20S proteasome (Baumeister et al., 1998) (Fig. 2).

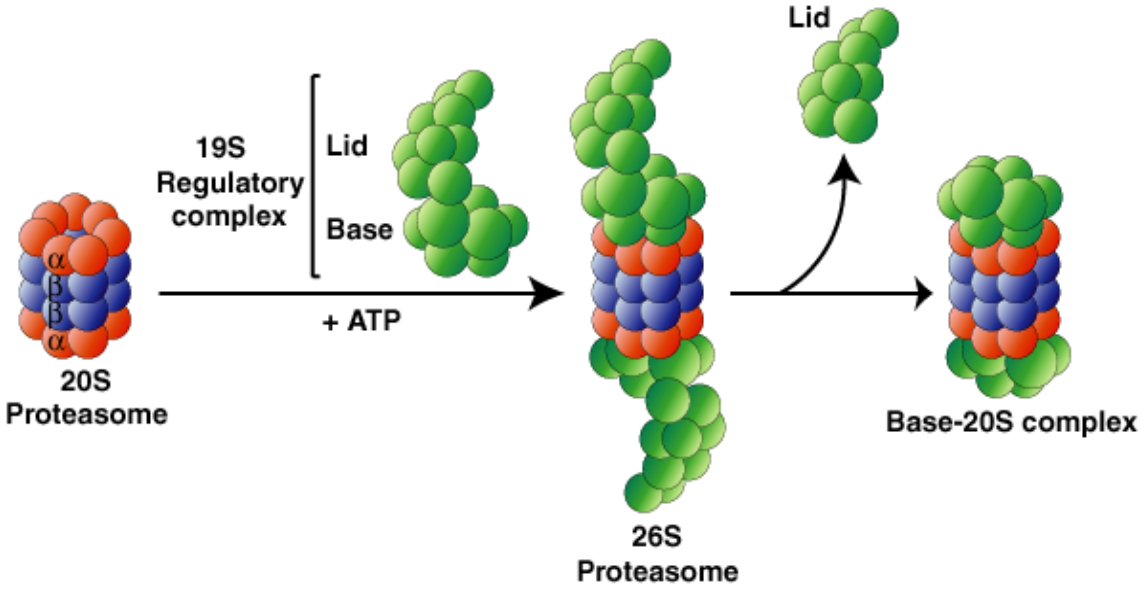



**Fig. 2. Structure of 26S proteasome**

The subunits of the 20S proteasome are classified into seven alpha subunits and seven beta subunits based on the similarity in the primary structure. The 20S proteasome consists of four stacked rings composed of alpha/beta/beta/alpha subunits. Target proteins pass through the narrow pores of the alpha rings and then are degraded by proteolytic beta subunits located inside the beta rings. Among the seven beta subunits of the 20S proteasome, only three subunits – beta1, beta2, and beta5 - are proteolytically active and have N-terminal catalytic threonine residues. These

"constitutive" subunits are also replaced with interferon-γ (IFN-γ)-inducible subunits, beta1i (also called LMP2), beta2i (also called LMP7), and beta5i (also called MECL1), respectively, in the immunoproteasome which plays a role in antigen processing and other immunological functions (Fruh and Yang, 1999; Kimura et al., 2015; Tanaka and Kasahara, 1998).

The 19S regulatory complex consists of six ATPase subunits (Rpt1-6) and thirteen non-ATPase subunits (Rpn1-3, Rpn5-15) (Tanaka, 1998). The 19S regulatory complex is also divided into two subcomplexes called "base" and "lid" (Glickman et al., 1998). The base complex consists of Rpt1-6, Rpn1, Rpn2, and Rpn13 while the lid complex consists of Rpn3, Rpn5-12, and Rpn15. The 19S regulatory complex plays a role in the regulation of proteasome function such as recognition of polyubiquitinated proteins, unfolding of target proteins, cleavage of polyubiquitin chains (i.e. isopeptidase activity), and regulation of the pores of the 20S proteasome (Ferrell et al., 2000). However, functions of each subunit have not been fully clarified. It has been also still unclear how the 26S proteasome recognizes various target proteins to degrade in a temporal and spatial manner.

## Functions and structure of Rpn10

Rpn10 is localized at the intermediate of the lid and the base complexes in the 19S regulatory complex and has been reported to be implicated in the recognition of polyubiquitinated proteins. Its N-terminal von Willebrand factor A (VWA) domain is considered to play a role in the interaction of those complexes (Fig. 3) (Glickman et al., 1998). On the other hand, its C-terminal region contains one or two polyubiquitin sites

(PUbS). Each PUbS contains a ubiquitin-interacting motif (UIM) which strongly binds with tetra or longer ubiquitin chains (Hofmann and Falquet, 2001). The UIMs consist of approximately 20 amino acids and contain a consensus sequence motif, xEDExLxxAxxxSxxExxxx (x is an arbitrary amino acid residue). It is considered that the bulky hydrophobic amino acid residues, acidic amino acid residues, and serine residue are important for the interaction of Rpn10 with polyubiquitin chains. A UIM was originally identified from Rpn10, but it has been found that other proteins, especially lysosomal proteolytic proteins, also have UIMs (Hofmann and Falquet, 2001). Although it is unknown if all the proteins containing UIMs could bind with polyubiquitin chains, it has been well validated that Rpn10 binds with polyubiquitinated proteins. Thus, Rpn10 is considered to be a ubiquitin receptor in the 26S proteasome.

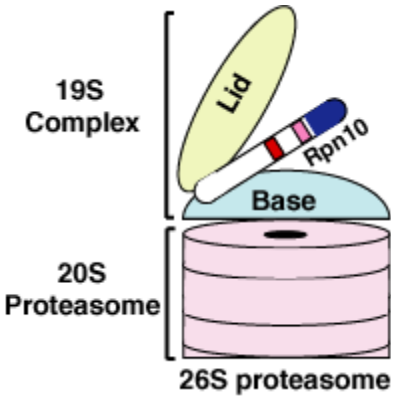



**Fig. 3. Localization of Rpn10**

Rpn10 orthologues have been identified to date in various organisms such as yeast, nematodes, fruit fries, vertebrates, and plants. They are also called Mcb1 in the moss *Physcimitrella patens* (Girod et al., 1999), Sun1 in the budding yeast *Saccharomyces cerevisiae* (van Nocker et al., 1996b), Pus1 in the fission yeast *Schizosaccharomyces pombe* (Wilkinson et al., 2000), Mbp1 in the grass plant *Arabidopsis thaliana*) (Deveraux et al., 1995), p-54 in the fruit fly *Drosophila melanogaster* (Haracska and Udvardy, 1995), and S5a in *Homo sapiens* (Ferrell et al., 1996), respectively. The structures and amino acid sequences of their N-terminal VWA domains are highly conserved. In yeast, Rpn10 (Sun1) was originally identified as one of the two multi-copy suppressors of *nin1-1* (the Rpn12 lethal mutation) (Kominami et

al., 1997). This effect was observed not only with the full length of Rpn10 but with the N-terminal 200 amino acids of Rpn10 (i.e. ΔPUbS mutant). These results suggest that Rpn10 plays an important role in construction of the 19S regulatory complex through its N-terminal region, interacting with other proteasome subunits and ligating the lid and the base complexes. In this regard, Rpn10 has been considered to interact with Rpn1 from the base complex and Rpn9, Rpn11, or Rpn12 from the lid complex (Ferrell et al., 2000). Moreover, a conserved-aspartate residue (Asp11) at the VWA domain of Rpn10 plays an important role in the formation of the 26S proteasome and it is known that a point-mutation of this amino acid results in disruption of the 26S proteasome (Fu et al., 2001).

In yeast, it has been reported that Rpn10 deficiency does not lead to lethality but exhibits enhanced sensitivity to amino acid analogues and has increased steady-state levels of ubiquitin-protein conjugates (van Nocker et al., 1996b; Wilkinson et al., 2000). Interestingly, this phenotype was rescued by expression of an Rpn10 mutant protein that lacks the UIM, suggesting that the C-terminal PUbS of yeast Rpn10 is dispensable and redundant for survival and the stress responses. On the other hand, *P. patens* Rpn10 (Mcb1) knockout (KO) strain suppressed its growth at early development and thus Mcb1 is found essential for normal growth (Girod et al., 1999). Interestingly, its N-terminal mutant caused more severe phenotype than its C-terminal mutant, suggesting the N-terminal domains of Rpn10 are important in yeast. This, however, may not be the case for vertebrate cells because the C-terminal structure of vertebrate Rpn10 is different from that of those organisms.

Most of the 26S proteasome subunits have highly-conserved structures among eukaryotes. However, the C-terminal structures of Rpn10 are largely different between

yeast and other organisms in the opisthokonta lineage. Yeast Rpn10 contains only one PUbS in the C-terminal region (van Nocker et al., 1996b; Wilkinson et al., 2000) while Rpn10 from other organisms generally contains two PUbS and other unique domain(s). This fact may suggest that Rpn10 acquired new functions in the extended C-terminal domains in the course of evolution in the opisthokonta lineage. For example, each PUbS in human Rpn10 contains a UIM but these two UIMs are not functionally identical; the UIM2 binds more strongly with polyubiquitin chains than the UIM1 *in vitro* (Young et al., 1998). Moreover, the human homolog of Rad23B (HR23B), a ubiquitin-like protein (UBL), has been reported to interact with PUbS2 but not with PUbS1 of human Rpn10 (S5a) (Hiyama et al., 1999). These results indicate distinct roles of two PUbS of Rpn10 in the recognition of polyubiquitinated target proteins and other functional proteins. Taken together, Rpn10 can interact with a wide variety of proteins and is considered to play various roles.

Kawahara et al. have identified five Rpn10 isoforms, Rpn10a, Rpn10b, Rpn10c, Rpn10d, and Rpn10e from *Mus musculus* (Fig. 4) (Kawahara et al., 2000a). All of them

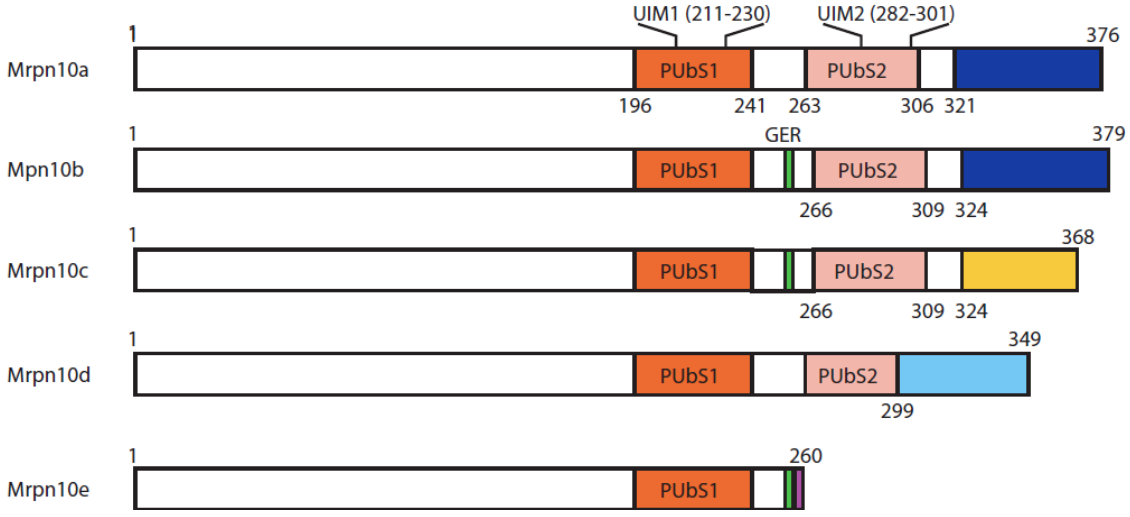



**Fig. 4. Schematic structures of mouse Rpn10 (Mrpn10) family**

are generated by alternative splicing of the introns of 3' regions of the sole gene. All the isoforms have the same N-terminal region but each isoform contains a distinct and unique structure in the C-terminal stretch. It has been speculated that Rpn10a is constitutively expressed and the others are highly expressed in mouse embryo. Rpn10e is a unique isoform in that its structure is similar to yeast Rpn10 and its expression reaches the peak in the embryonic brain at late development stages. Thus, it is speculated that there may be different types of the 26S proteasome which contain different Rpn10 isoforms, depending on developmental stages, possibly playing a role in proteolysis of different types of proteins. The specific function of each Rpn10 isoform, however, has been almost unknown to date. It has been also totally unknown if other species than mice and humans have a variety of Rpn10 splicing isoforms.

## Objective of my studies

To shed the light on the significance of Rpn10 isoforms, I studied genomic organization of Rpn10 gene from various organisms including nematodes, fish, and frogs in the first chapter. In addition, to clarify the role of an Rpn10 isoform in the 26S proteasome, I focused on *Xenopus* Rpn10c, identified a specific interacting-protein, and investigated its physiological role in the *Xenopus* development in the second chapter.

# Chapter 1:

# The 26S Proteasome Rpn10 Gene Encoding Splicing Isoforms: Evolutional Conservation of the Genomic Organization in Vertebrates

## Abstract

Recognition of polyubiquitinated substrates by the 26S proteasome is a key step in the selective degradation of various cellular proteins. The Rpn10 subunit of the 26S proteasome can bind to polyubiquitin conjugates *in vitro*. Kawahara et al (2000a). previously reported the unique diversity of Rpn10, which differs from other multiple proteasome subunits, and that the mouse Rpn10 mRNA family is generated from a single gene by developmentally-regulated alternative splicing. To determine whether such alternative splicing mechanisms occur in other species, I searched for Rpn10 isoforms in databases and in my original PCR products.

Here I report the genomic organization of Rpn10 gene in lower vertebrates and provide evidence for the competent generation of distinct forms of Rpn10 by alternative splicing through evolution. My results suggested that the diversity of Rpn10 caused by alternative splicing has been acquired during the course of vertebrate evolution, or that such splicing potential was abandoned in invertebrates.

## Introduction

Ubiquitin is a covalent modifier that produces a polyubiquitin chain acting as a selective degradation signal (Coux et al., 1996; Hershko and Ciechanover, 1998; Hochstrasser, 1996). Protein ubiquitination is catalyzed by a multi-enzymatic system consisting of E1 (ubiquitin-activating), E2 (ubiquitin-conjugating), and E3 (ubiquitin-ligating) enzymes. Most E3 enzymes directly bind to target proteins in a substrate-specific manner, in a process designed to select proteins for degradation (Hershko and Ciechanover, 1998). There is ample evidence in support of the critical role of the ubiquitin pathway in a variety of biologically important processes, including cell-cycle control, apoptosis, signal transduction, development, and immune response (Hershko and Ciechanover, 1998; Kawahara et al., 2000b; Kawahara and Yokosawa, 1992; Kornitzer and Ciechanover, 2000; Schwartz and Ciechanover, 1999; Tanaka and Kasahara, 1998; Tanaka and Kawahara, 2000).

Degradation of polyubiquitinated proteins is catalyzed by the 26S proteasome, a eukaryotic ATP-dependent protease complex (Voges et al., 1999). The 26S proteasome is composed of a core proteinase complex, known as the 20S proteasome (Bochtler et al., 1999), and a pair of symmetrically disposed PA700 regulatory particles (alias the 19S complexes) (Baumeister et al., 1998; Coux et al., 1996; Ferrell et al., 2000; Kanayama et al., 1992). PA700 is attached to both ends of the central 20S proteasome in opposite orientations to form the enzymatically active 26S proteasome in an ATP-dependent manner (DeMartino and Slaughter, 1999; Tanahashi et al., 1999).

PA700 was discovered as an activator of the 20S proteasome (DeMartino et al., 1996; DeMartino and Slaughter, 1999). It is a 700-kDa protein complex composed of

~20 subunits each ranging in size from 25 to 110 kDa (DeMartino and Slaughter, 1999; Tanaka, 1998). PA700 consists of two subcomplexes, known as “base” and “lid”, which, in the 26S proteasome, correspond to the portions of PA700 proximal and distal, respectively, to the 20S proteasome (Glickman et al., 1998). The base is comprised of six ATPases and two largest regulatory components named Rpn1 and Rpn2, while the lid contains multiple non-ATPase subunits. The base-complex, thought to bind to the outer α-ring of the central 20S proteasome ATP-dependently, is thought to be involved in opening the gate of the α-ring for entry of the protein substrate, as well as chaperon-like activity toward substrates for unfolding, so substrates can be penetrated into a chamber formed by the β-ring where the active sites are located (Braun et al., 1999). On the other hand, the lid-complex is thought to be required for recognition of target proteins. The lid- and base-complexes assemble to form the regulator complex PA700 through multiple subunit interactions (Fu et al., 2001; Glickman et al., 1998).

Recognition of polyubiquitinated substrates by the 26S proteasome is a key step for the selective degradation of various cellular proteins. Previous studies showed that the Rpn10 subunit of human PA700, originally called S5a, can bind to a polyubiquitin chain linked to proteins *in vitro* (Deveraux et al., 1994; Deveraux et al., 1995). Moreover, deletional analyses of Rpn10 revealed that there are at least two independent polyubiquitin binding sites, named UIM1 and UIM2 (also called PUbS1 and PUbS2, respectively), in the carboxyl terminal half of human Rpn10 (Fu et al., 1998; Haracska and Udvardy, 1997; Hofmann and Falquet, 2001; Young et al., 1998). Although only one segment (i.e., UIM1) appears to be sufficient for the polyubiquitin chain-binding activity as seen in yeast Rpn10 or the segment artificially produced from Rpn10 of other organisms (Fu et al., 1998; Girod et al., 1999; Haracska and Udvardy,

1997), the co-existence of UIM2 increases apparent affinity for polyubiquitin chains, indicating that both UIM1 and UIM2 act in concert for polyubiquitin recognition *in vitro* (Young et al., 1998). In mammals, both UIM1 and UIM2 are approximately 30-amino acid long and are separated by 21 intervening amino acid residues (Young et al., 1998). It is interesting to note that the length between two UIM motives is strictly conserved in vertebrates, although amino acid sequences of the intervening regions are not strongly conserved, unlike the high conservation in these UIM regions.

Rpn10 homologues have been identified in various eukaryotes, such as yeast (Kominami et al., 1997; van Nocker et al., 1996b), plant (van Nocker et al., 1996a), fruit fly (Haracska and Udvardy, 1995), moss (Girod et al., 1999), fish (Yanagawa et al., 1998), mice (Kawahara et al., 2000a; Pusch et al., 1998), and humans (Ferrell et al., 1996), and are given various names; such as Mcb1, Sun1, Mbp1, p-54 and S5a. Curiously, the size of Rpn10 is exceptionally small in only the yeast protein lacking the C-terminal region beyond the UIM1 domain (Fig. 5) (Kominami et al., 1997; van Nocker et al., 1996b), indicating that the C-terminal additional domains including UIM2 and KEKE, a domain enriched in alternating Lys and Glu (Realini et al., 1994), have been acquired in multicellular organisms or abandoned in yeast during evolution in the opisthokonta lineage.

As reported previously, the mouse Rpn10 mRNA family is generated from a single gene by developmentally-regulated alternative splicing (Fig. 6) (Kawahara et al., 2000a). Mouse Rpn10 gene is about 10 kbp-long, which is comprised of 10 exons, and specific sequences of variant Rpn10 family-proteins are encoded in intronic regions of the Rpn10a gene present ubiquitously in all cells (Kawahara et al., 2000a). However, it has been unknown whether Rpn10 family-proteins exist in other species than mice.

In this chapter, I sought to clarify genomic organization of Rpn10 gene from various organisms and determined the genomic sequences encoding Rpn10 from nematodes, fish and frogs. Comparison of the genomic and cDNA sequences of Rpn10 revealed similar gene organizations from lower vertebrates to human, suggesting potential existence of Rpn10 isoforms in vertebrates, although actual splicing products have not been cloned from other vertebrates than mammals. Taken together, highly-conserved Rpn10 genomic structure in vertebrates suggests its significant and the fundamental role of the Rpn10 family.

## Materials and Methods

*Determination of Rpn10 gene sequences of Oryzias latipes and Xenopus laevis*

First, the partial sequences of the coding regions of the Rpn10 genes were amplified by degenerated PCR from *O. latipes* ovary cDNA libraries and *X. lavis* cDNA libraries prepared from embryos using Takara Ex Taq (Takara Bio Inc.) The primer sequences used in the experiments were 5'-CTGGCTTTGCGTGT(G/C)TCIATG-3' and 5'-GTTCTCAAGCACICT(T/C)TG-3' for *O. latipes* and 5'-CTGGCTTTGCGTGT(G/C)TCIATG-3' and 5'-GATTCTCCAGGACICT(T/C)TG-3 for *X. laevis*. Next, the 5'-terminal and 3'-terminal of the Rpn10 genes were amplified by nested PCR. The PCR primer sequences used for *Oryzias latipes* Rpn10 were 5'-CTGGCTTTGCGTGTGTCGATG-3' and 5'-AATTAACCCTCACTAAAGGG-3' for the first PCR for the 5'-terminal sequences, 5'-AAGACGCCCTGTTGAAGATG-3' and 5'-CGCTCTAGAACTAGTGGATC-3' for the second PCR for the 5'-terminal sequences, 5'-CCTGCATCACATCGTAGTCG-3' and 5'-GTAAAACGACGGCCAGT-3' for the first PCR for the 3'-terminal sequences, 5'-ATCTGCTCCTCCTCGGTCAT-3' and 5'-TCGAGGTCGACGGTATC-3' for the second PCR for the 3'-terminal sequences. The PCR primer sequences used for *X. laevis* Rpn10 were 5'-CTGGCTTTGCGTGTGTCGATG-3' and 5'-AATTAACCCTCACTAAAGGG-3' for the first PCR for the 5'-terminal sequences, 5'-GGAGGAGCAGAGGCAGCGGCA-3' and 5'-CGCTCTAGAACTAGTGGATC-3' for the second PCR for the 5'-terminal sequences, 5'-ATTCTCCAGGACGCTCTGTAA-3' and 5'-GTAAAACGACGGCCAGT-3' for the first PCR for the 3'-terminal sequences, 5'-AAGAACTCGGGGTCCTGCATC-3' and 5'-TCGAGGTCGACGGTATC-3' for the

second PCR for the 3'-terminal sequences. Finally, the whole sequences of Rpn10 genome and Rpn10a cDNA were amplified by PCR from the cDNA libraries described above, *O. latipes* genome DNA libraries or *X.* genomic DNA prepared from testis. The PCR primer sequences were 5'-ATGGTGCTTGAAAGTACTAT-3' and 5'-TCACTTTTTTTTCTCCTCTT-3' for *O. latipes* Rpn10, and 5'-ATGGGGCTGGAAAGTACTAT-3' and 5'-TCATTTCTTCTCCTCCTTCTT-3' for *X. laevis* Rpn10. Amplified PCR products were cloned into pGEM-T Easy Vector (Promega Inc.) Sequencing reactions used T7 and Sp6 primers and ABI BigDye version 2.0 chemistry (Applied Biosystems). Sequences were determined using an ABI PRISM 377 Genetic Analyzer.

Human and rat Rpn10 isoforms were identified based on the expressed sequence tag (EST) sequences found from the NCBI Nucleotide Database (https://www.ncbi.nlm.nih.gov/nuccore) by using the amino acid sequences of mouse Rpn10 isoforms as a query.

## Results

To determine whether alternative splicing of Rpn10 could have occurred in other species than mice, I searched for the existence of Rpn10 isoform transcripts in the EST database and applied PCR amplification in my analysis. My studies identified the existence of homologous splice variants not only in mice but also in other mammals including humans and rats (Table 1). These genes contain homologous sequences specific for each Rpn10 family, which are spliced at exactly the same position of the gene, suggesting that these mRNAs for the Rpn10 family in each species are generated by a universal mechanism, at least in mammals. In accordance with this observation, the genomic structures of mouse and human Rpn10 genes are quite identical, although the length of introns is somewhat diverse. In contrast, so far I have not found any Rpn10 isoforms in EST databases of non-vertebrate species. To compare the organizations of invertebrate Rpn10 genes with those of the vertebrates, I searched the public database for genomic sequences. The available genomic databases revealed the lack of intronic sequences in the region corresponding to the C-terminal half in nematode, *Caenorhabditis elegans*, and fruit fly, *Drosophila melanogaster*, strongly indicating that any Rpn10-splicing isoforms are not produced in these organisms (Fig. 5). This means that the diversity of Rpn10 caused by alternative splicing has been acquired during the course of vertebrate evolution, or that such splicing potential was abandoned in invertebrates.

To determine whether alternative splicing of Rpn10 is specific to mammals, I isolated and determined the sequence of genomic clones and cDNA clones encoding Rpn10 from Japanese rice fish, *O. latipes*, as an example of the lower vertebrates.

Comparison of the genomic and cDNA sequences of Rpn10 revealed similar gene organizations extending from the fish to human (Fig. 5 and 6). The positions of the exon-intron junctions and the length of each exon are also completely conserved among vertebrates Rpn10 genes (Fig. 5). This finding implies that the competence for all distinct forms of Rpn10 alternative splicing is widely retained in vertebrates. Although actual splicing products have not yet been reported to date, my results indicate that some of the Rpn10 isoforms are in fact expressed in lower vertebrates (shown and discussed in the next chapter). In considering the fact that mouse Rpn10 mRNA family is generated from a single gene by developmentally regulated, alternative splicing (Kawahara et al., 2000a), the strong conservation of Rpn10 genomic structure in diverse vertebrates indicates its significant and the fundamental role of the Rpn10 family in the development of vertebrates.

## Discussion

In general, almost all proteasome subunits are highly conserved in eukaryotes (Tanaka, 1998). However, recent studies have shown that certain proteasome genes have been acquired or diverged around an era close to the origin of vertebrates. For example, it is known that a major immunomodulatory cytokine IFN-γ induces replacement of three constitutive β-type subunits of the 20S proteasome by IFN-γ-inducible subunits, thereby producing “immunoproteasomes” responsible for immunological processing of intracellular antigens, which play a critical role in adaptive immunity (Tanaka and Kasahara, 1998). Furthermore, IFN-γ also induces a proteasome activator known as PA28, REG or the 11S regulator, consisting of two related subunits, α and β, which appears to be involved in the accelerating processing of certain antigens (DeMartino and Slaughter, 1999; Hendil et al., 1998; Murata et al., 1999; Rechsteiner et al., 2000; Tanaka and Kasahara, 1998). Naturally the genome of yeast lacking immunity does not contain genes coding for IFN-γ-inducible genes. These genes all appear to have emerged close to the origin of vertebrates to accommodate the needs for production of antigenic peptides capable of binding efficiently to major histocompatibility complex class I molecules (Tanaka and Kasahara, 1998). Likewise, the existence of multiple forms of Rpn10 in mammals and perhaps other vertebrates, but the apparent absence of Rpn10 splice variants in invertebrates, may represent another example of adaptive changes in evolution, indicating that Rpn10 has species-specific functions in higher eukaryotes.

The alteration of structural complexities in the Rpn10 subunit is presumably responsible for acquisition of its functional changes. Intriguingly, it has been

documented that human Rpn10a interacts directly with various other proteins. For example, the Ub-like domain of hHR23 (the human homologue of yeast Rad23) can interact directly with the region of UIM2 in the C-terminal half of Rpn10a (Hiyama et al., 1999). Thus, the evolutionarily acquired C-terminal diversity of the Rpn10 family expands the possibility of Rpn10-mediated interactions of potential proteasome substrates and proteolytic regulators. Therefore, it is not surprising that the phenotypes of Rpn10 deficiency in several organisms are rather inconsistent depending on species. Indeed, disruption of the gene orthologous to Rpn10 (*rpn10*) did not cause any obvious defects in yeast (Kominami et al., 1997; van Nocker et al., 1996b), but intriguingly Rpn10 suppression induced meiotic arrest and an F2 sterile phenotype in *C. elegans* (Shimada et al., 2006), and gene knockout (KO) of Rpn10 by homologous recombination was associated with fetal death in mice (Hamazaki et al., 2007). Thus, unlike the situation in yeast, Rpn10 appears to play a more important role in reproduction and embryonic development in other eukaryotes.

One fundamental function of Rpn10 is assistance in establishing a linkage between the base and lid complex, and formation of the stable 19S complex (Glickman et al., 1998). In addition, the polyubiquitin binding ability by UIM1 domain in the Rpn10 protein is thought to be redundant rather than essential (Fu et al., 1998). In considering evolution of the *RPN10* gene to vertebrates, my favorite scenario is that the Rpn10 molecule has acquired or retained its C-terminal extension and complexities generated by the mechanism of alternative splicing, which meets the requirement for expanded diversity of ubiquitin-mediated protein degradation. Further functional analyses of Rpn10 isoforms are needed to determine the significance of complexities of the Rpn10 family. Clarification of the substrate-recognition diversity by the Rpn10

family will be a future prospect.

## Tables and Figures

**Table 1. Mammalian Rpn10 isoforms.**

| | Rpn10a | Rpn10b | Rpn10c | Rpn10d | Rpn10e |
|---|---|---|---|---|---|
| Mouse | NM008951 | AW541266 | AA060854 | AB029145 | BI687156 |
| Rat | AF175575 | AB017188 | AI236731 | ND | Direct cloning |
| Human | U72664 | AL570579 | BI006619 | ND | AB033605 |

These Rpn10 variants shown by EST accession numbers are found in the EST database. Some isoforms were identified by my RT-PCR-based amplification techniques. ND, not determined.

**Fig. 5. Schematic representation of Rpn10 protein structures of various evolutionary distinct species.**

The positions of introns in their corresponding genome sequences are marked as indicated by the symbol. The polyubiquitin-binding domains (UIM1 and UIM2), C-terminal highly conserved sequence (CS) and the KEKE domain are boxed as indicated by filled designs. Yeast, *Saccharomyces cereviciae*, *Schizosaccharomyces pombe*; nematode, *Caenorhabditis elegans*; fruit fly, *Drosophila melanogaster*; Japanese rice fish, *Oryzias latipes*; mouse, *Mus musculus*; and human, *Homo sapiens*.

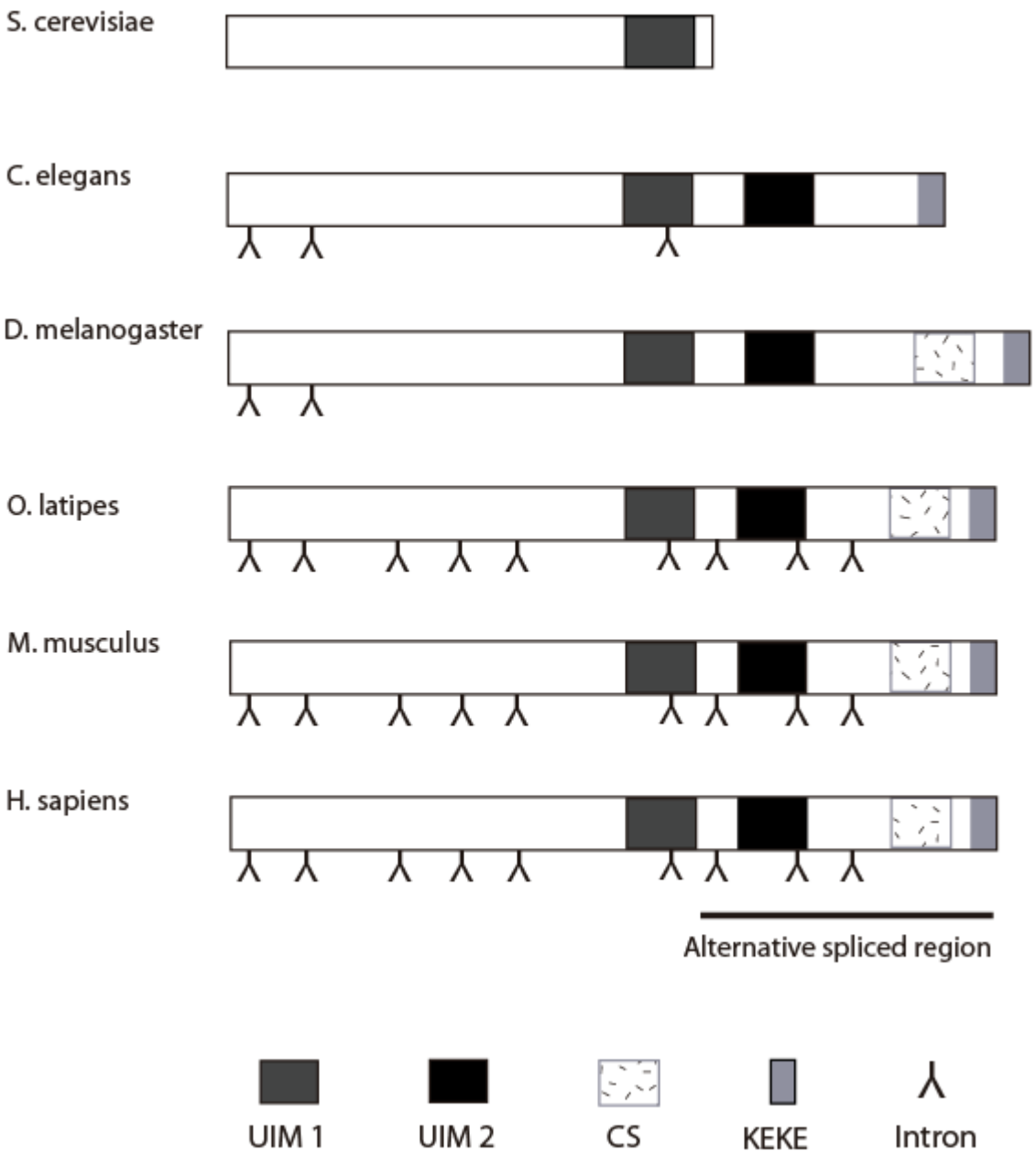
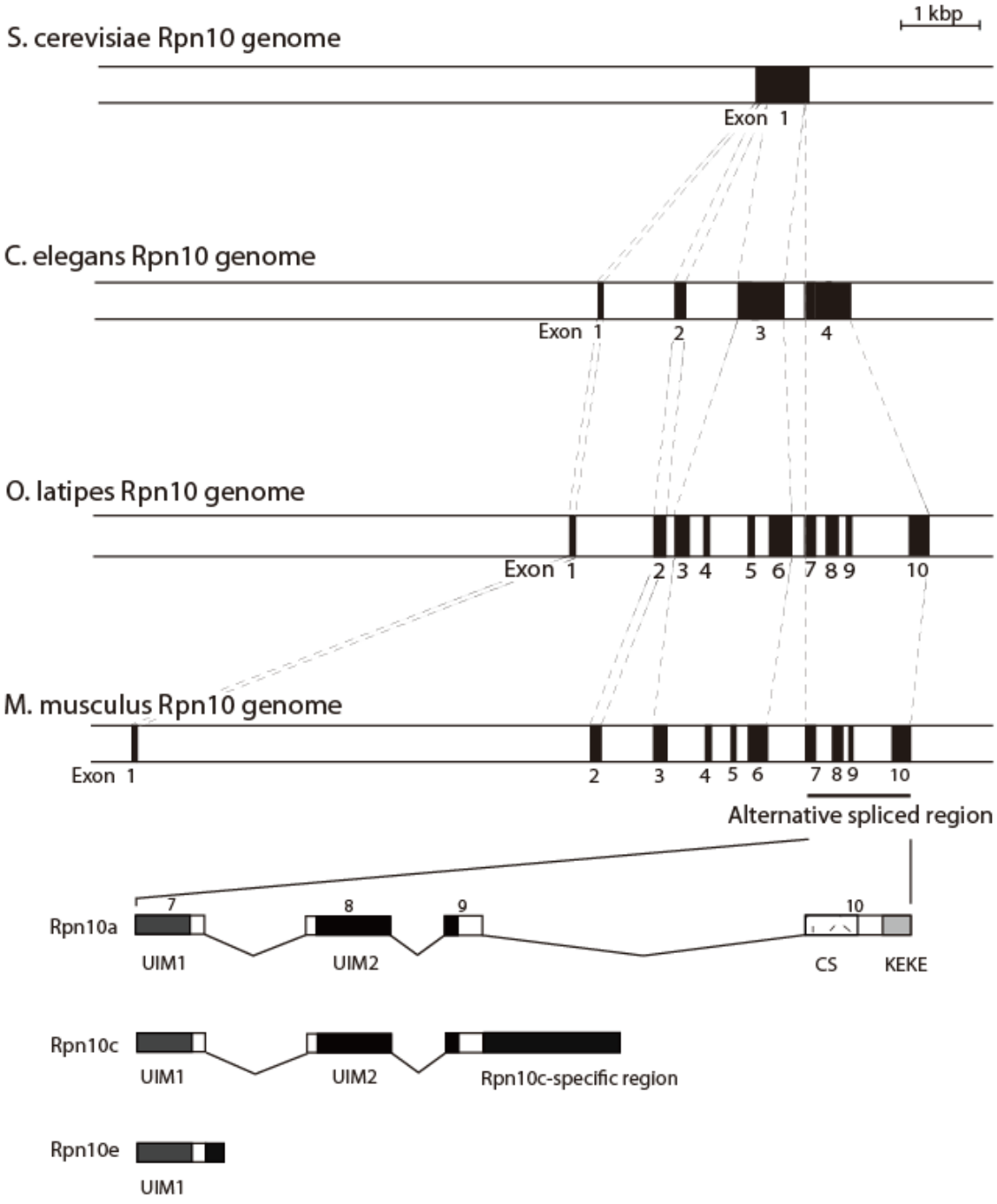

S. cerevisiae
C. elegans
D. melanogaster
O. latipes
M. musculus
H. sapiens
Alternative spliced region
UIM 1
UIM 2
CS
KEKE
Intron

**Fig. 6. Physical maps of genomic organization of yeast, nematode, fish and mouse Rpn10 genes.**

The scale shows the length of 1 kbp. Exons are indicated by solid boxes and numbered from 1 to 10. The structures of the mouse multiple Rpn10 transcripts generated by alternative splicing are shown schematically at the bottom of the figure.
Protein-encoding regions are represented by *boxes* and introns by *lines*. Solid boxes: UIM1, UIM2 or Rpn10c-specific regions, as specifically indicated. Open boxes: less-conserved regions.


1 kbp
S. cerevisiae Rpn10 genome
Exon 1
C. elegans Rpn10 genome
Exon 1 2 3 4
O. latipes Rpn10 genome
Exon 1 2 3 4 5 6 7 8 9 10
M. musculus Rpn10 genome
Exon 1 2 3 4 5 6 7 8 9 10
Alternative spliced region
Rpn10a
7 8 9 10
UIM1 UIM2 CS KEKE
Rpn10c
UIM1 UIM2 Rpn10c-specific region
Rpn10e
UIM1

# Chapter 2:

# Unique Proteasome Subunit Xrpn10c Is a Specific Receptor for the Antiapoptotic Ubiquitin-like Protein Scythe

## Abstract

The Rpn10 subunit of the 26S proteasome can bind to polyubiquitylated and/or ubiquitin-like proteins via the ubiquitin-interacting motifs (UIMs). The vertebrate Rpn10 consists of five distinct spliced isoforms, but the specific function of these variants remains largely unknown. I demonstrated that one of the alternative products of *Xenopus* Rpn10, named Xrpn10c, functions as a specific receptor for Scythe/BAG-6 that has been reported to regulate Reaper-induced apoptosis. Deletional analyses revealed that Scythe has at least two distinct domains responsible for its binding to Xrpn10c. Conversely, an Xrpn10c has an UIM-independent Scythe binding site. The forced expression of a Scythe mutant protein lacking Xrpn10c-binding domains in *Xenopus* embryos induces inappropriate embryonic death, while the wild-type Scythe did not show any abnormality. These results indicate that Xrpn10c-binding sites of Scythe act as an essential segment linking the ubiquitin/proteasome machinery to the control of proper embryonic development.

## Introduction

Ubiquitin is a covalent modifier that produces a polyubiquitin chain that functions as a degradation signal (Finley et al., 2004; Hershko and Ciechanover, 1998; Hershko et al., 2000; Schwartz and Hochstrasser, 2003). Degradation of polyubiquitylated proteins is catalyzed by the 26S proteasome, a eukaryotic ATP-dependent protease complex (Baumeister et al., 1998; Coux et al., 1996; DeMartino and Slaughter, 1999; Voges et al., 1999). The 26S proteasome is composed of the catalytic 20S proteasome and a regulatory complex termed PA700 or 19S complex. PA700 is a 700-kDa protein complex composed of six ATPase subunits (Rpt1-6) and multiple non-ATPase subunits (Rpn1-3, Rpn5-15), each ranging in size from 11 to 110 kDa (DeMartino and Slaughter, 1999; Tanaka, 1998).

Recognition of polyubiquitylated substrates by the 26S proteasome is a key step in the selective degradation of various cellular proteins (Pickart, 1997; Pickart and Cohen, 2004; Szlanka et al., 2003). Previous studies have shown that several ubiquitin-associated (UBA) domain proteins and the Rpn10 subunit of 26S proteasome, originally called S5a, can bind to a polyubiquitin chain linked to proteins *in vitro* (Elsasser et al., 2004; Ferrell et al., 1996; Haracska and Udvardy, 1997; van Nocker et al., 1996b; Verma et al., 2004; Wilkinson et al., 2001). A deletional analysis of Rpn10 revealed that there are at least two independent polyubiquitin-binding sites, named UIM1 (PUbS1) and UIM2 (PUbS2), in the C- terminal half of vertebrate Rpn10 (Hofmann and Falquet, 2001; Young et al., 1998). Although only one segment (i.e., UIM1) appears to be sufficient for polyubiquitin chain-binding activity as was found in yeast Rpn10 (Beal et al., 1998; Elsasser et al., 2004; van Nocker et al., 1996b), the

co-existence of UIM2 increases the affinity for binding of polyubiquitin chains, indicating that both UIM1 and UIM2 act in concert for polyubiquitin recognition *in vitro* (Young et al., 1998). In addition to polyubiquitin chain binding, it has been shown that UIM2 of human Rpn10 interacts with several ubiquitin-like (UBL) proteins via their UBL domains. For example, the UBL domains of hHR23B (the human homologue of yeast Rad23) and PLIC (the human homologue of yeast Dsk2) can directly interact with human Rpn10 (Hiyama et al., 1999; Schauber et al., 1998; Walters et al., 2002). Thus, mammalian Rpn10 is thought to be one of the recognition sites for several UBL proteins, as well as for polyubiquitin chains.

It was previously reported that the mouse *rpn10* mRNA family is generated from a single gene by developmentally-regulated alternative splicing, producing Rpn10a to Rpn10e (Kawahara et al., 2000a). The mouse *rpn10* gene is about 10 kbp long and is composed of 10 exons, and it has been found that specific sequences of variant Rpn10 family proteins are encoded in the intronic regions of the *rpn10a* gene, suggesting that the repertoire of the mouse *rpn10* mRNA family is regulated at the post-transcriptional level (Kawahara et al., 2000a). Rpn10a is an ortholog of human S5a (Haracska and Udvardy, 1997) and is ubiquitously expressed during development, while Rpn10c is specifically expressed in mouse embryonic tissues and at particularly high levels in ES cells (Carter et al., 2003; Kawahara et al., 2000a). Rpn10c contains two UIM domains as is the case with Rpn10a, but it also contains a unique sequence in its C-terminal region differing from any other proteins including other Rpn10 isoforms. However, apart from its characteristic expression pattern, the role of Rpn10c is not known at present.

Apoptosis is a form of cell death and is essential for the correct development and homeostasis of multicellular organisms (Chinnaiyan and Dixit, 1996; Hensey and Gautier, 1998; Stack and Newport, 1997). A potent apoptotic inducer, Reaper, is critical for programmed cell death in the fruit fly *Drosophila melanogaster* (White et al., 1994). Although Reaper homologues in other species have not yet been reported, it has been shown that ectopic expression of Reaper in human cells and in *Xenopus* cell-free extracts can also trigger apoptosis, suggesting that Reaper-responsive pathways are conserved (Thress et al., 1999; Thress et al., 1998). Thress et al. (1998) identified a 150-kDa protein as the Reaper-binding molecule in *Xenopus* egg extracts and designated this protein as Scythe (Thress et al., 1998). It has been reported that Scythe contains a BAG domain as a chaperone-binding region in its C-terminal region (and thereby also called BAG-6) and a single UBL domain in its N-terminal region, but the function of the latter domain remains completely elusive to date.

To investigate the function of the Rpn10c subunit of 26S proteasomes, I cloned the *Xenopus* counterpart of mouse Rpn10c cDNA and named the gene *xrpn10c*. Here I report that Xrpn10c protein is a specific receptor of Scythe/BAG-6. I found that an Xrpn10c-specific C-terminal sequence is required and sufficient for Scythe binding. Conversely, I identified novel tandem domains in the N-terminal region of Scythe and found that these domains are necessary for Xrpn10c binding. I also found that forced expression of a Scythe mutant lacking Xrpn10c-binding sites induced inappropriate embryonic development. These findings provide the first evidence that N-terminal tandem domains of Scythe act as essential regions linking the ubiquitin/proteasome machinery to the control of *Xenopus* embryonic development.

## Materials and Methods

*Plasmid construction*

The full-length cDNAs of Xrpn10a, Xrpn10c and Scythe were amplified by PCR from *Xenopus* cDNA libraries prepared from stage 25 embryos. To generate a series of Xrpn10 expression vectors, PCR products were cloned into the pCR2.1 vector, respectively (Invitrogen, San Diego, CA). After digestion with *Eco*RI and *Sal*I, each *xrpn10* isoform gene was inserted into the pCI-neo-Flag mammalian expression vector (Promega, Madison, WI). Similarly, the PCR products of Scythe subcloned into the pCR2.1 vector were digested with *Sal*I and *Not*I and inserted into the pCI-neo-T7 vector. The truncated and mutated versions of Xrpn10 and Scythe were constructed by PCR with pCI-neo vectors as templates using a forward primer and mutated reverse primers. The Xrpn10 (N5) mutants were generated using a QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) and subcloned into the pCI-neo-Flag vector. The GFP-fused expression vectors of Xrpn10 were constructed by digesting pCI-neo-T7-Xrpn10 with *Eco*RI and *Sal*I, and the resulting fragment was subcloned into pEGFP-C2 (Clontech Laboratories, Palo Alto, CA). Sequences of all plasmids were verified before transfection experiments.

*Immunoprecipitation and immunoblotting*

COS7 cells (monkey kidney cell line) were transiently transfected with the indicated plasmids using FuGENE6 (Roche Molecular Systems, Inc., NJ) according to the protocol supplied by the manufacture. The total amount of plasmid DNA was adjusted to 1 μg with an empty vector. After incubation for 36 h, the cells were

harvested and subjected to immunoprecipitation and/or Western blot analyses. After the cells had been washed with ice-cold PBS, they were lysed with a buffer containing 50 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 0.5% Triton X-100, complete protease inhibitor cocktail (Roche), 10 mM N-ethylmaleimide and 50 μM MG132 (Peptide Institute Inc. Tokyo, Japan). The cell lysate was sonicated for 10 seconds, and the debris was removed by centrifugation at 13,000 × g for 20 min. The resulting supernatant was incubated with anti-Flag M2 affinity gel (Sigma Chemical Co., St. Louis, MO) for 2 h at 4°C, and the immunocomplex produced was washed five times with lysis buffer. Immunoprecipitation of the 26S proteasome was conducted using an antibody specific for the Rpt6 ATPase subunit of the human 26S proteasome (Hendil et al., 1998) and Protein A-Sepharose 4B (Amersham Biosciences, Uppsala, Sweden).

For Western blotting, the whole cell lysate and immunoprecipitates were separated by SDS-PAGE and transferred onto nitrocellulose membranes (Bio-Rad, Richmond, CA). The membranes were immunoblotted with anti-T7 (Novagen, Madison, WI), anti-Myc (9E10, Santa Cruz Biotechnology, Santa Cruz, CA), anti-Flag M2 (Sigma) and anti-green fluorescent protein (Clontech) antibodies and then incubated with horseradish peroxidase-conjugated antibody against mouse or rabbit immunoglobulin (Amersham Biosciences, Buckinghamshire, UK), followed by detection with ECL Western blotting detection reagents (Amersham Biosciences, UK).

*GST pull-down assay*

For expressing GST-fusion proteins, all genes were subcloned into the pGEX6P1 vector (Amersham Pharmacia) and transformed into *E. coli BL21* (DE3). GST-fusion proteins were expressed in *E. coli*, and the extracts were applied to

glutathione-immobilized agarose beads (Amersham Pharmacia) and eluted with 50 mM glutathione in 50 mM Tris-HCl, pH 8.0. The eluted proteins were then dialyzed against buffer A (50 mM Tris-HCl, pH 7.5, containing 1 mM dithiothreitol, 150 mM NaCl, 0.1% TritonX-100, and 10% glycerol). Then glutathione-immobilized beads in the same buffer were added to an equal volume of the above reaction mixture and incubated for 2 h at 4°C. After extensive washing, the proteins that had bound to beads were used for GST pull-down experiments.

For preparation of non-tagged recombinant Xrpn10 proteins or Scythe Domain I and Domain II fragments, the beads were suspended in an appropriate volume of buffer A containing PreScission protease (Amersham Biosciences), and the mixture was incubated for 12 h at 4°C to allow the protease to cleave the GST-tag. The proteins thus formed were then used as purified Xrpn10 proteins. Purified non-tagged proteins and GST-fusion proteins coupled with beads were mixed, incubated, and precipitated, and the resulting pull-down samples were subjected to Western blotting with appropriate antibodies as indicated.

*RT-PCR*

For RT-PCR analysis, *Xenopus* embryos were disrupted by treatment with TRIzol (Life Technologies, Inc., Gaithersburg, MD), and total RNAs were extracted. Five μg of total RNA was then reverse-transcribed with SUPERSCRIPT II reverse transcriptase (Life Technologies, Inc.) using random hexamers. Using the cDNA products as templates, *xrpn10* cDNAs were amplified by PCR with primers specific for *xrpn10a* and *xrpn10c*. Twenty-five cycles for *xrpn10a* or 30 cycles for *xrpn10c* were

run with denaturation at 94°C for 1 min, annealing at 65°C for 1 min, and elongation at 72°C for 5 min.

*Expression of proteins in Xenopus embryos*

Full-length cDNAs for Xrpn10a, Xrpn10c and Scythe were subcloned into the RN3 vector (Lemaire et al., 1995), and the mRNAs were synthesized *in vitro* by mMESSAGEmMACHINE (Ambion Inc., Austin, TX). The synthesized mRNAs were dissolved in RNase-free water, and 5 ng of mRNAs was injected in a volume of 9.2 nL into a blastomer of 2-cell stage embryos of *Xenopus* embryos. Embryos were cultured in a 0.2 × MMR solution at 20°C. At the blastulae stage, each embryo was individually harvested, crushed in PBS, and centrifuged to collect the cytoplasm fraction. Samples of this fraction were used for immunoprecipitations with an anti-Flag antibody and subsequently subjected to Western blot analysis.

*Nucleotide sequence*s

The nucleotide sequence data reported in this paper appear in the DDBJ, EMBL and GenBank Nucleotide Sequence Databases with the following accession numbers: *Xrpn10* genome (AB190306), *Xrpn10a* cDNA (AB190304), *Xrpn10c* cDNA (AB190305).

## Results

### Identification of *xrpn10c* in *Xenopus* embryos

The mouse *rpn10* gene is comprised of 10 exons, and specific retention of several introns generates multiple spliced isoforms, including at least five distinct forms, named Rpn10a to Rpn10e (Kawahara et al., 2000a). Comparison of the genomic sequences revealed identical exon-intron organizations of *rpn10* genes in all of the vertebrates examined (Fig. 7A). These findings imply that the competence for all distinct forms of *rpn10* alternative splicing is conserved among vertebrates.

*Rpn10c*, one of the spliced forms of the *rpn10* gene, was originally isolated from mouse ES cells and has been detected in mouse embryonic tissues (Kawahara et al., 2000a). As a model system for further developmental analysis, I looked at *rpn10* family transcripts in the African clawed frog, *Xenopus laevis*, and found that the *rpn10c* homologue is adequately expressed in the developing *Xenopus* embryos. PCR-assisted cloning allowed us to isolate the full-length cDNA encoding the *Xenopus* counterpart of *rpn10c* as well as a universally expressed *rpn10a* homologue, and I designated these genes as *xrpn10c* and *xrpn10a*, respectively (Fig. 7B). Sequence alignment of Xrpn10a and Xrpn10c revealed that they have identical sequences in their N-terminal halves, including two UIM segments, whereas the C-terminal region varied greatly (Fig. 7B). The C-terminal region of Xrpn10c contains a unique sequence that shows no overall homology to the sequences of other known proteins except for its orthologues in vertebrates (Fig. 7B, C). In Xrpn10c-specific C-terminal extensions, I identified a relatively conserved amino acid stretch, and I tentatively designated this region as 10c-box (Fig. 7C). The expression profile of *xrpn10* family genes was analyzed by

reverse transcription-polymerase chain reaction (RT-PCR) using a set of primers corresponding to the specific sequences of either the *xrpn10a* or *xrpn10c* gene (Fig. 7D). The *xrpn10a* transcript was found to be expressed constitutively from unfertilized eggs to adult tissues, indicating its ubiquitous expression, as is the case with the mouse *rpn10a*. In contrast, using primers A and C, fragments of 580-bp were amplified exclusively from embryonic stages 15 to 25 and no detectable expression was observed in unfertilized eggs and earlier embryos. Sequence analysis of these fragments confirmed that the 580-bp band indeed corresponds to *xrpn10c*. Thus, *xrpn10c* was found to be a transcript whose expression is altered in a developmental stage-specific manner.

**Xrpn10c specifically binds to Scythe, a UBL protein**

To explore the roles of Xrpn10c, I searched for a protein(s) that specifically interacts with Xrpn10c. Since it has been reported that several UBL domain proteins can interact directly with the C-terminal half of mammalian S5a/Rpn10a (Hiyama et al., 1999; Walters et al., 2002), I cloned several UBL protein genes from a *Xenopus* cDNA library and examined their interactions with Xrpn10 family proteins. I confirmed that both XHR23B and XDRP1 (Funakoshi et al., 1999), *Xenopus* counterparts of yeast Rad23 and Dsk2, respectively, could bind equally to both Xrpn10a and Xrpn10c in a UIM domain-dependent manner (Fig. 8). In contrast, Scythe was exclusively co-immunoprecipitated with Xrpn10c, while there was no interaction between Xrpn10a and Scythe (Fig. 9). It has been reported that Scythe is composed of an N-terminal single UBL domain and a C-terminal BAG domain as well as intervening repetitive

sequences (Thress et al., 1998; Thress et al., 2001). These results indicate that Scythe is a UBL protein that specifically interacts with Xrpn10c.

Since both Xrpn10c and Scythe are proteins that are expressed in *Xenopus* embryos, I carried out an experiment to determine whether Xrpn10c can interact with Scythe in developing *Xenopus* embryos. I microinjected *in vitro* synthesized mRNA encoding Xrpn10c and Scythe into the fertilized eggs of *X. laevis* and harvested the embryos at the blastulae stage (stage 7). The mRNA injection resulted in production of corresponding proteins in the *Xenopus* embryos (Fig. 10A, input). It was found that Xrpn10c, but not Xrpn10a, specifically precipitated with Scythe (Fig.10A, IP), as was the case in extracts of COS7 cells (Fig. 9). These results indicate that Xrpn10c protein can associate with Scythe in the developing *Xenopus* embryos. I also found that the exogenously expressed Xrpn10c protein, as well as Xrpn10a, was incorporated into the endogenous 26S proteasome complex in living embryos, since immunoprecipitation with antibody against Rpt6, an ATPase subunit of the endogenous 26S proteasome, simultaneously co-precipitated Xrpn10c and Xrpn10a (Fig. 10B, IP). I do not know the reason why the incorporation of Flag-Xrpn10a seems to be much lower than that of Xrpn10c. As there are no good antibodies specific for Xrpn10c, it has not been possible to demonstrate the presence of endogenous Xrpn10c proteins in 26S proteasomes.

Using anti-Scythe antibody, it was found that there is no detectable binding of endogenous Scythe to proteasome at this early developmental stage (Fig. 10B, IP left lane). Only if Flag-Xrpn10c mRNA is injected, endogenous Scythe can be adequately co-immunoprecipitated with 26S proteasomes (Fig. 10B, IP center lane), but not if the Xrpn10a version is overexpressed (Fig. 10B, IP right lane). In the former case, the amount of Xrpn10c containing proteasome versus Xrpn10a proteasomes might be

increased significantly, whereas in the latter case, the putatively large population of Xprn10a proteasomes could stay unchanged or increase only slightly. All this is in favor for specific binding of Scythe to Xrpn10c and not to Xrpn10a in the context of the 26S proteasome components.

**Xrpn10c-specific region functions as a novel site for Scythe recognition**

To identify the Scythe-binding site in Xrpn10c, I co-expressed a series of Flag-tagged Xrpn10c mutant proteins and T7-tagged Scythe (Fig. 11). I found that the C-terminal half of Xrpn10c was necessary for Scythe binding (Fig. 11A, D). Remarkably, mutational analysis revealed that neither the UIM1 nor the UIM2 domain is necessary for Scythe binding (Fig. 11B, D). These results indicate that Scythe interacts with Xrpn10c by a mechanism different from those in the cases of other known UBL proteins such as hHR23A/B. Further deletion analysis of Xrpn10c revealed that a segment containing the Xrpn10c-specific region was necessary and sufficient for Scythe binding (Fig. 11B, C, D). The most critical region for Scythe binding in Xrpn10c was the C-terminal region containing amino acid residues 331 to 339 (Fig. 11C, D), designated as 10c-box (Fig. 7C), the sequences of which are conserved across species, and deletion of this sequence largely abolished Scythe binding (Fig. 11B, C, D). To precisely evaluate the contribution of the 10c-box sequence for Scythe binding, I quantified the relative intensity of immunosignals of 10c-box-lacking forms of Xrpn10c compared to 10c-box-including forms. The signal of Xrpn10c (1-330) decreased more than 89% compared to that of (1-339). Similarly, the signal of (249-330) decreased more than 71% compared to that of (249-339), and the signal of (304-330) decreased more than 78% compared to that of (304-339). Consistent with the importance of the

10c-box sequence, a GST-fusion protein with the 10c-box consisting of nine amino acids could bind with Scythe as strongly as the full-length Xrpn10c (discussed later in Fig. 13B, C). These results indicate that the 10c-box is directly responsible for the interaction of Xrpn10c with Scythe.

**Novel tandem UBL domains of Scythe contribute to Xrpn10c binding**

To identify the Xrpn10c-binding site in Scythe, I generated T7-tagged deletion mutants of Scythe protein and co-expressed them with Flag-tagged Xrpn10 in COS7 cells. I found that a segment containing the N-terminal region (1-436) was sufficient for Xrpn10c binding, indicating that the BAG domain at the C-terminus of Scythe is not necessary for Xrpn10c binding (Fig. 12A, B). In good agreement with these *in vivo* observations, an *in vitro* GST pull-down assay using recombinant proteins suggests a direct interaction between Xrpn10c and N-terminal fragment of Scythe (Fig. 13A). Xrpn10c, but not Xrpn10a, was co-precipitated with GST-Scythe (1-436) (the N-terminal 436-amino acid fragment of Scythe; designate as N436), while neither GST-Scythe (801-1113) (the C-terminal 313-amino acid fragment of Scythe; designate as C313) nor GST alone precipitated Xrpn10c (Fig. 8A), indicating that the N-terminal region of Scythe is required for its direct binding with Xrpn10c.

Unexpectedly, deletion of N-terminal UBL domain (86 amino acids) from full-length and N-terminal 436-amino acid fragment of Scythe did not abolish Xrpn10c-binding. My further analysis revealed that, within N436 fragment, there are two independent segments called Domain I (Scythe 1-214) and Domain II (Scythe 215-436) that can bind with Xrpn10c *in vivo* (Fig. 12A, B). Results of *in vitro* GST pull-down assays using recombinant proteins also suggest that Xrpn10c or its 10c-box

peptide directly interact with the fragment of either Domain I (Fig. 13B) or Domain II (Fig. 13C) of Scythe protein. Domain I contains a typical UBL domain (amino acid residues 7-81; 38.2% identity and 64.5% similarity to ubiquitin) as reported by Thress et al. (Thress et al., 1998) (see Fig. 14A) in its N-terminus, and this UBL sequence in the Domain I was essential for Domain I to bind with Xrpn10c (Fig. 12A, B). On the other hand, no ubiquitin homology had been reported in the region corresponding to the Domain II. However, my close inspection of the primary sequence revealed that N-terminal half of Domain II indeed contains an additional sequence with a homology to ubiquitin (amino acid residues 257-323; 26.3% identity and 46.1% similarity to ubiquitin), and I designate this region here as UBL2 (Fig. 14A. C). Note that I designated the UBL motif in the N-terminus of Domain I as UBL1 to distinguish it from UBL2. It is important to note that the region of UBL2 is essential for Domain II to interact with Xrpn10c (Fig. 14B, C). Thus, the results of my analysis suggested the presence of a novel second ubiquitin homology sequence that had not been previously identified and show that ubiquitin homology domains in both Domain I and Domain II are involved in targeting of Scythe to Xrpn10c *in vivo*. These results indicate that Scythe is a novel protein that contains functional tandem ubiquitin homology sequences in its N-terminal region.

**Tandem UBL domains contributes to the function of Scythe**

Scythe was originally identified as a novel anti-apoptotic protein, though the function of its UBL domain remains entirely obscure (Thress et al., 1998). In fact, expression of the N-terminal truncated form of Scythe (ΔN100) lacking UBL1 did not have any effect on normal *Xenopus* development. To address the significance of my

finding that Scythe contains unique tandem ubiquitin homology domains that are required for Xrpn10c interaction, I synthesized translatable mRNAs encoding T7-tagged Scythe and a series of its UBLs-truncated mutant proteins, and then injected the respective mRNAs into a blastomere of 2-cell stage embryos.

It has been reported that the C312 fragment of Scythe is a potent, Reaper-independent inducer of apoptosis in a *Xenopus* cell-free system (Thress et al., 1998). Recombinant Scythe C312 protein induced apoptotic nuclear fragmentation and caspase DEVDase activation with a time course similar to that for Reaper-induced apoptosis in the extracts (Thress et al., 1998). I confirmed these results by my *in vivo* assay by injecting mRNA encoding Scythe C312 into a blastomere of 2 cell-stage embryos, which resulted in complete impairment of normal tadpole development (Fig. 15A). The expression of full-length Scythe (FL) did not influence normal development (Fig. 15). Neither expression of ΔUBL1 (in which amino acid residues 7-81 had been deleted from the full length of Scythe) nor that of ΔUBL2 (in which amino acid residues 258-324 had been deleted) caused detectable developmental abnormality (Fig. 15A). In contrast, the expression of Scythe protein lacking both UBL1 and UBL2 (ΔUBL1, 2; simultaneous deletion of amino acid residues 7-81 and 258-324) triggered inappropriate embryonic development and greatly reduced the rate of normal tadpole development (Fig. 15). Embryos expressing Scythe (ΔUBL1, 2) underwent rounds of normal cell division during their blastula stage, but they progressively deviated from normal morphogenesis thereafter and failed to develop into normal tail bud embryos. These results suggest that the UBL1 and UBL2 domains of Scythe are redundantly involved in the control of appropriate progression of embryogenesis during the course of *Xenopus* development.

## Discussion

In this study, I found that proteasomal Xrpn10c subunit physically associates with Scythe in *Xenopus* embryos, while there is no interaction between Scythe and Xrpn10a, a ubiquitous form of Rpn10 splicing variants (Kawahara et al., 2000a). Xrpn10c has a unique extension at the C-terminal side. I found that an Xrpn10c-specific C terminal sequence is required and sufficient for Scythe binding. The essential region of Xrpn10c for Scythe binding is amino acid residues 331-339, and I called this motif 10c-box. Although 10c-box does not have obvious sequence similarity to other UBL binding domains, such as UIM, I concluded that Xrpn10c containing the 10c-box functions as a Scythe binding receptor. I suggest that the region containing the 10c-box is a novel candidate for the UBL protein-binding domain of the 26S proteasome. It has not yet been determined whether this motif can interact with other known UBL proteins in general. Alternatively, it is plausible that the 10c-box is a binding motif specific to tandem ubiquitin homology domain of Scythe, because XHR23B and XDRP1 did not interact with 10c-box (Fig. 8).

In yeast, it has been reported that UBL domains of Rad23 and Dsk2 bind the leucine-rich-repeat (LRR)-like region in Rpn1 of the 26S proteasome (Elsasser et al., 2002; Seeger et al., 2003), indicating that Rpn1 is a general receptor for the UBL domain. In addition to Rpn1, UIMs of the Rpn10 subunit have also been identified as alternative acceptor sites for UBL domains of hHR23A/B, PLIC and Parkin in vertebrates (Hiyama et al., 1999; Sakata et al., 2003). These results collectively indicate that there are multiple acceptor sites for specific classes of UBL proteins in the 26S proteasome complex. The existence of distinct binding sites for UBL proteins on the

26S proteasome might ensure simultaneous interactions between several UBL proteins and the 26S proteasome, preventing competition among them. In addition, it is of note that mammalian Rpn10 gene generates multiple variants through alternative splicing, which may contribute to the achievement of functional diversity of 26S proteasomes with their respective isoforms. In this regard, it is interesting that Rpn10c exhibits a unique interaction with Scythe. The unanswered question is whether different physiological binding partners have varying receptor preferences and, if so, what features of substrates might predispose them to a particular docking mode. Thorough analysis of changes of proteasome function in mutants that possess defects in the respective interactions will be necessary to elucidate this point.

Scythe was originally identified as a binding protein of Reaper, a potent apoptotic inducer, and was suggested to inhibit Reaper-induced apoptosis in *Xenopus* egg extracts (Thress et al., 1998). It has been reported that the BAG domain of Scythe regulates Hsp70-mediated protein folding and that Scythe-mediated inhibition of Hsp70 is reversed by Reaper (Thress et al., 2001). Although the role of the N-terminal UBL domain has not been elucidated, it has been reported that the addition of the C-terminal fragment of Scythe (Scythe C312) in *Xenopus* egg extracts induced Reaper-independent apoptosis (Thress et al., 1999; Thress et al., 1998), implying the potential role of Scythe N-terminal half in the regulation of apoptosis. In this paper, I identified two distinct domains in the N-terminal region of the Scythe, Domain I and Domain II, capable of binding Xrpn10c redundantly. Domain I contains a typical UBL sequence (designated here UBL1), as reported by Thress et al. (Thress et al., 1998), and I found that deletion of this UBL1 region abolished the binding ability of Domain I for Xrpn10c. Domain II also contained a UBL2 sequence with similarity to ubiquitin, which had not been

reported previously. UBL2 is comprised of 67 amino acid residues, displaying 46% and 41% overall similarity to ubiquitin and UBL1, respectively (Fig. 9A), and this region is well conserved in the mammalian homologue of Scythe called BAT3. I found that UBL2 is an essential sequence within Domain II for the association with Xrpn10c. Thus, it can be concluded that Scythe is a novel protein with at least two tandem ubiquitin homology domains, UBL1 and UBL2. It is of note that these ubiquitin homology domains of Scythe did not interact with the UIM of Rpn10 and Rpn1 subunit of 26S proteasome, differing from other UBL-containing proteins. Unexpectedly, I found that both UBL1 and UBL2 domains are necessary but not themselves sufficient for interaction with Xrpn10c. This finding indicates that both domains require the respective additional C-terminal regions in Domain I and Domain II, respectively, to interact with Xrpn10c and implies that the UBL domains, together with their additional C-terminal sequences, form novel structures that associate with a domain unrelated to UIM or UBA domains. Further structural analyses are in progress.

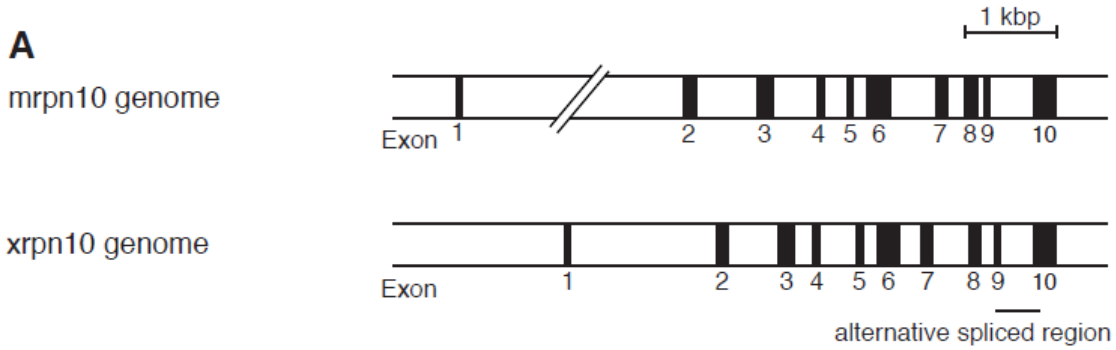
Scythe belongs to a family of BAG proteins (Takayama and Reed, 2001; Takayama et al., 1995). It has been reported that BAG-1 is the physical link between the Hsc70/Hsp70 chaperone system, ubiquitination machineries and the proteasome (Alberti et al., 2002; Demand et al., 2001; Luders et al., 2000; Takayama et al., 1997). In a way similar to the case with BAG-1, it is possible that Scythe links the proteasomes to chaperones. Indeed, the UBL regions of Scythe are associated with the Xrpn10c subunit of the 26S proteasome, while the C-terminal BAG domain combines the molecular chaperones Hsp70 (Thress et al., 1999; Thress et al., 2001). My preliminary analysis indicated that Scythe was co-precipitated with Xchip, a *Xenopus* homologue of the chaperone-dependent E3 ubiquitin ligase CHIP (C-terminus of Hsc70-interacting

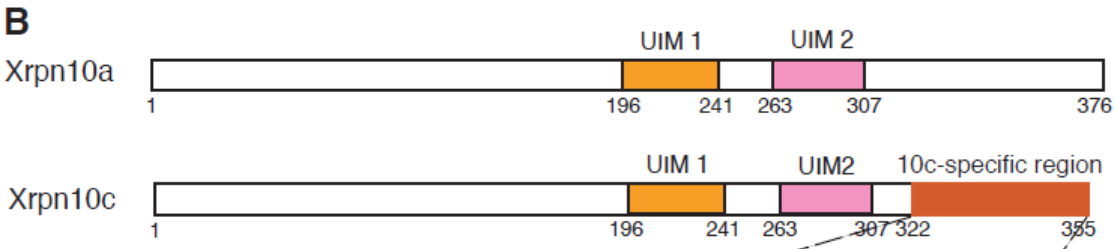
protein) (Murata et al., 2001; Wiederkehr et al., 2002). My findings imply that Xrpn10c and Scythe may act as novel physical coupling factors to form a multi-complex comprising the 26S proteasome, the molecular chaperone Hsp70 and the E3 ubiquitin ligase. Furthermore, it was reported that the UBL/UBA domain proteins Rad23 and PLIC act as adaptor molecules in the control of post-ubiquitination events (Elsasser et al., 2002; Kleijnen et al., 2000). My results imply that UBL/BAG adaptor proteins recognize chaperone substrates and deliver them to the proteolytic machinery. Although such protein(s) of the apoptotic pathway is currently obscure, the results of the present study suggest that substrate recognition occurs by temporally and spatially regulated expression of Xrpn10 isoforms in collaboration with specific UBL proteins. Thus, targeting of substrates to the 26S proteasome might be regulated by multiple mechanisms. Accordingly, further studies are required to clarify the substrate-recognition diversity of UBL proteins and Rpn10 family proteins.

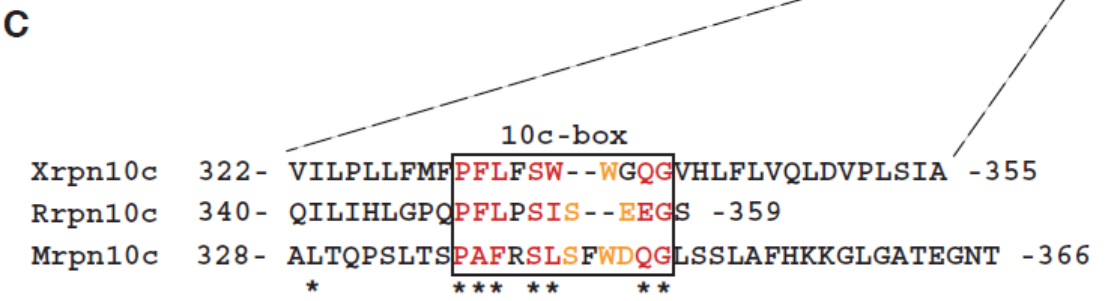
## Figures

**Fig. 7. Identification of the *xrpn10c* gene from *Xenopus*.**

(A) Physical maps of genomic organization of the *Xenopus* rpn10 gene (*xrpn10*). The scale shows the length of 1 kbp. Exons are indicated by filled boxes and numbered from 1 to 10. The exon-intron structure of *xrpn10* is identical to that of the mouse *rpn10* gene (*mrpn10*). The alternatively retained intron for generating *xrpn10c* is marked "alternative spliced region" (for details, see Kawahara et al., 2000). (B) Schematic representation of the structures of Xrpn10a and Xrpn10c proteins deduced from cDNA sequences. The ubiquitin-interacting domains (UIM1 and UIM2) and Rpn10c-specific region are indicated by colored boxes. (C) Alignment of C-terminal sequences of Rpn10c proteins from Xenopus (Xrpn10c), rat (Rrpn10c) and mouse (Mrpn10c). The conserved region (amino acid residues 331-340) is indicated by the open box and designated '10c-box'. (D) Expression of *xrpn10c* mRNA is developmentally regulated. PCR primers were designed for the conserved sequence in UIM1 (primer A), *xrpn10a*-specific region (primer B) and *xrpn10c*-specific region (primer C). RT-PCR was performed using the mRNA derived from embryos of the respective stages of development (right panel).


A
1 kbp
mrpn10 genome
Exon 1 2 3 4 5 6 7 8 9 10
xrpn10 genome
Exon 1 2 3 4 5 6 7 8 9 10
alternative spliced region


B
Xrpn10a
UIM 1
UIM 2
1 196 241 263 307 376
Xrpn10c
UIM 1
UIM2
10c-specific region
1 196 241 263 307 322 355


C
10c-box
Xrpn10c 322- VILPLLFMFPFLFSW--WGQGVHLFLVQLDVPLSIA -355
Rrpn10c 340- QILIHLGPQPFLPSIS--EEGS -359
Mrpn10c 328- ALTQPSLTSPAFRSLSFWDQGLSSLAFHKKGLGATEGNT -366
* *** ** **

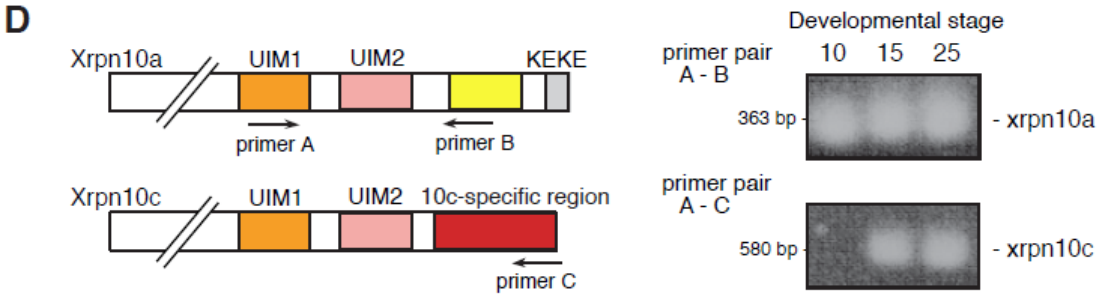

D
Xrpn10a
UIM1
UIM2
KEKE
primer A
primer B
Xrpn10c
UIM1
UIM2
10c-specific region
primer C
Developmental stage
primer pair A - B
10 15 25
363 bp
- xrpn10a
primer pair A - C
580 bp
- xrpn10c

**Fig. 8. Xrpn10a and Xrpn10c interact with XHR23B and XDRP1 in a UIM domain-dependent manner.**

Various deletion mutants of Flag-tagged Xrpn10a and Xrpn10c were expressed in COS7 cells. Cell extracts were mixed with GST protein or GST-fusion proteins of XHR23B and XDRP1, and the mixture was subjected to an *in vitro* GST pull-down assay with glutathione-Sepharose beads. The precipitants were immunoblotted with anti-Flag antibody. UIM1 (N5), UIM2 (N5) and UIM1,2 (N5) indicate site-directed substitution of the core sequences of UIM1, UIM2 and both to successive five Asn residues (LALAL for UIM1 and IAYAM for UIM2 to NNNNN, respectively). Xrpn10a,c (1-312), which retains UIM1 and UIM2 but lacks variable C-terminal region, interacted with XHR23B and XDRP1 while Xrpn10c Δ196-307, which retains the 10c-box but lacks UIM1 and UIM2, did not bind to XHR23B and XDRP1.

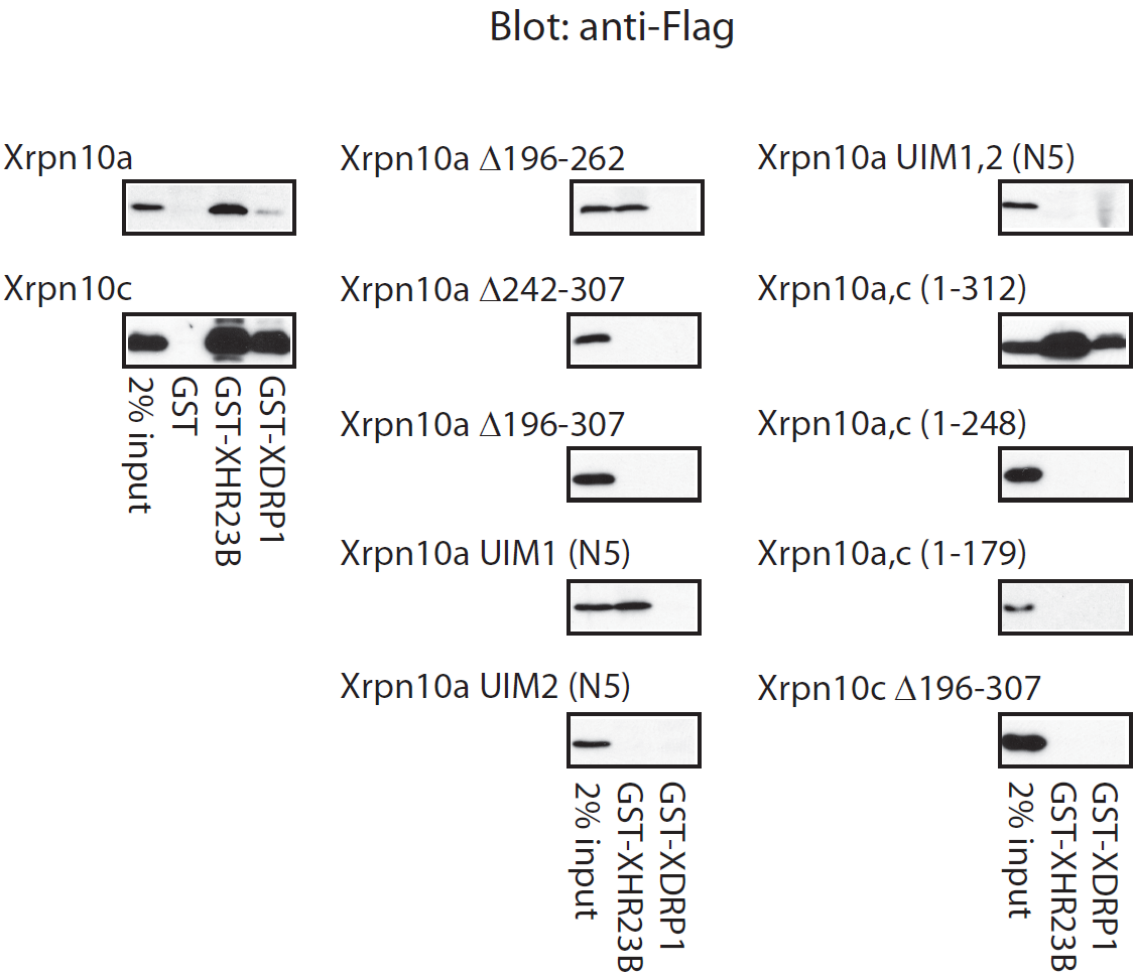

Blot: anti-Flag
Xrpn10a
Xrpn10c
2% input
GST
GST-XHR23B
GST-XDRP1
Xrpn10a Δ196-262
Xrpn10a Δ242-307
Xrpn10a Δ196-307
Xrpn10a UIM1 (N5)
Xrpn10a UIM2 (N5)
Xrpn10a UIM1,2 (N5)
Xrpn10a,c (1-312)
Xrpn10a,c (1-248)
Xrpn10a,c (1-179)
Xrpn10c Δ196-307

**Fig. 9. Xrpn10c, but not Xrpn10a, interacts with Scythe.**

T7-tagged Scythe and Flag-tagged Xrpn10a or Xrpn10c were expressed in COS7 cells at the indicated combinations. Cell extracts were immunoprecipitated with anti-Flag M2 agarose beads, and the precipitates were immunoblotted with anti-T7 and anti-Flag antibodies.

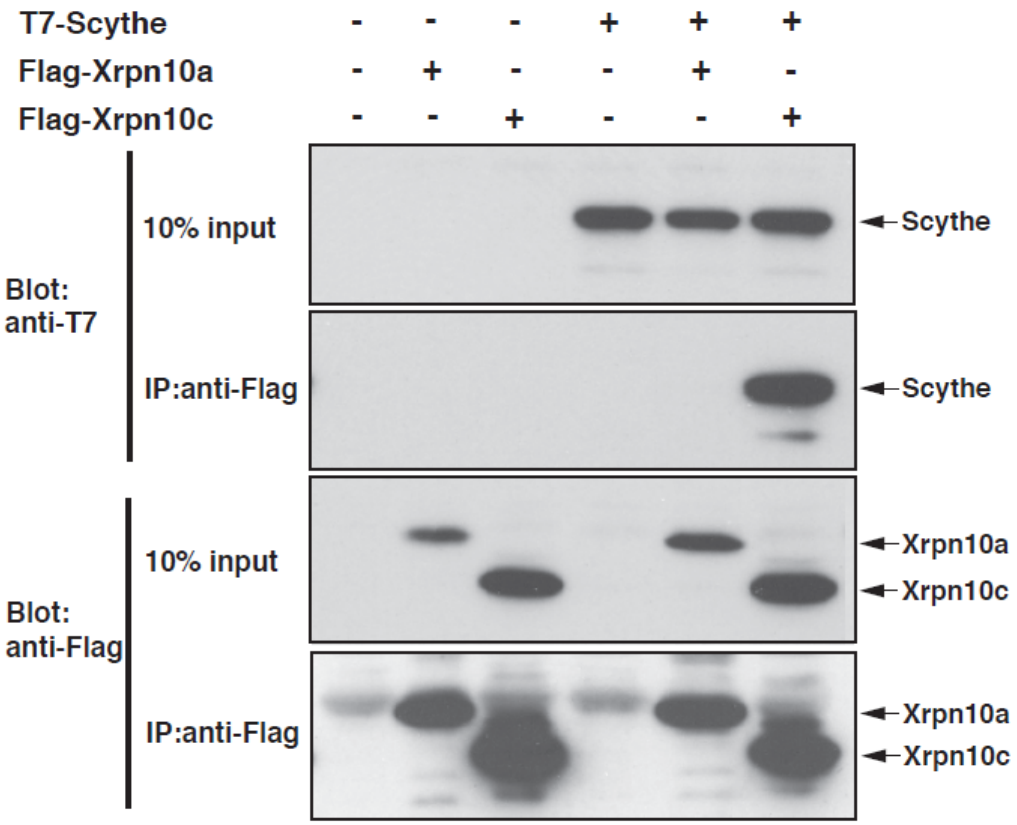

T7-Scythe - - - + + +
Flag-Xrpn10a - + - - + -
Flag-Xrpn10c - - + - - +
Blot: anti-T7
10% input
IP:anti-Flag
Scythe
Scythe
Blot: anti-Flag
10% input
IP:anti-Flag
Xrpn10a
Xrpn10c
Xrpn10a
Xrpn10c

**Fig. 10. Xrpn10c interacts with Scythe and the 26S proteasome in *Xenopus* embryos.**

Synthetic mRNAs for Flag-Xrpn10a and Xrpn10c were microinjected into fertilized eggs of *X. laevis*, and the embryos were harvested at the blastulae stage for immunoprecipitation analysis. (A) T7-tagged Scythe was co-precipitated with Flag-tagged Xrpn10c but not with Xrpn10a from *Xenopus* embryonic extracts. (B) Both Flag-tagged Xrpn10a and Xrpn10c were co-immunoprecipitated with the endogenous proteasomes by antibody against Rpt6 ATPase subunit of the 26S proteasome. Endogenous Scythe protein was also co-precipitated by antibodies against Rpt6 and 20S proteasome complex in the condition of Xrpn10 expression.

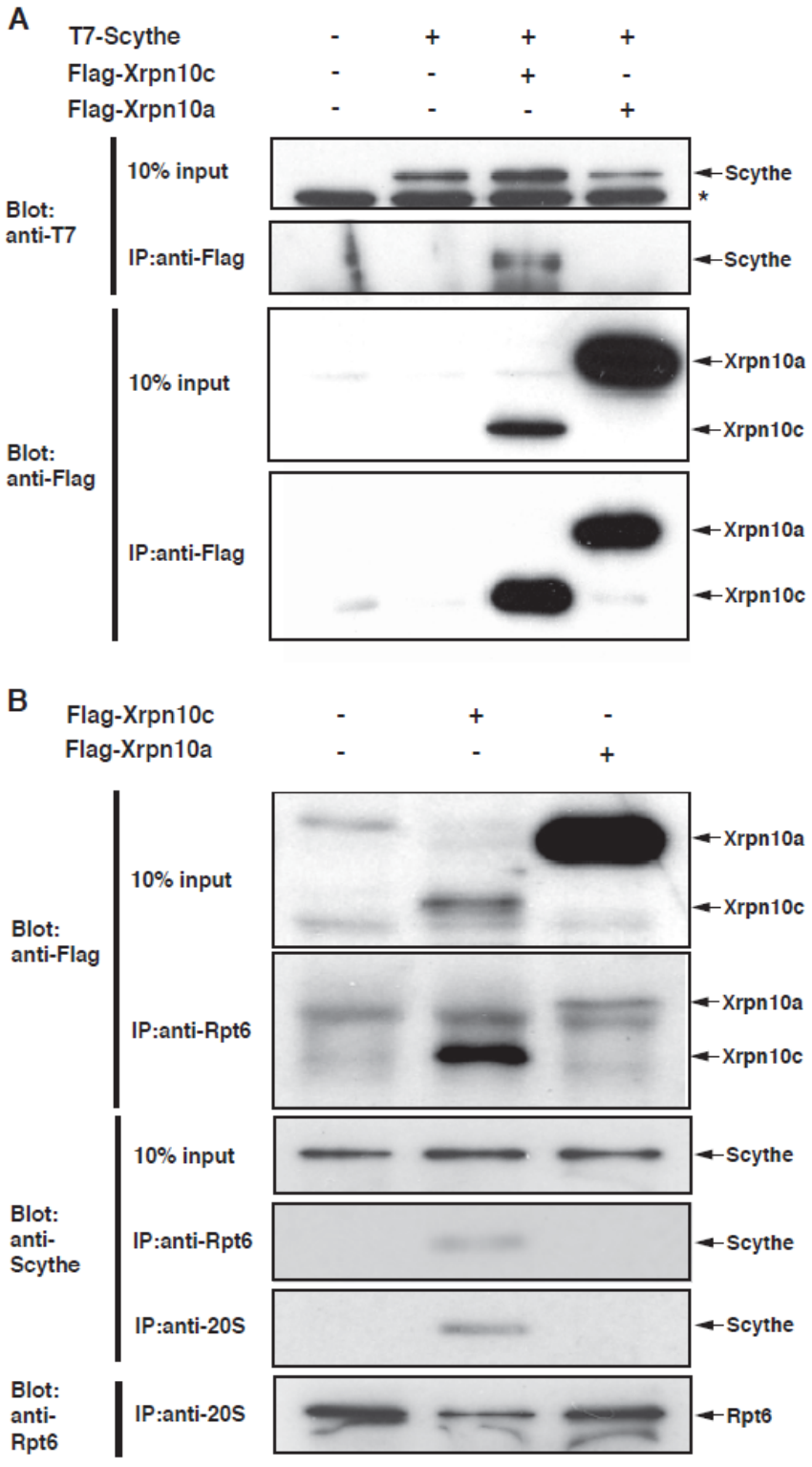
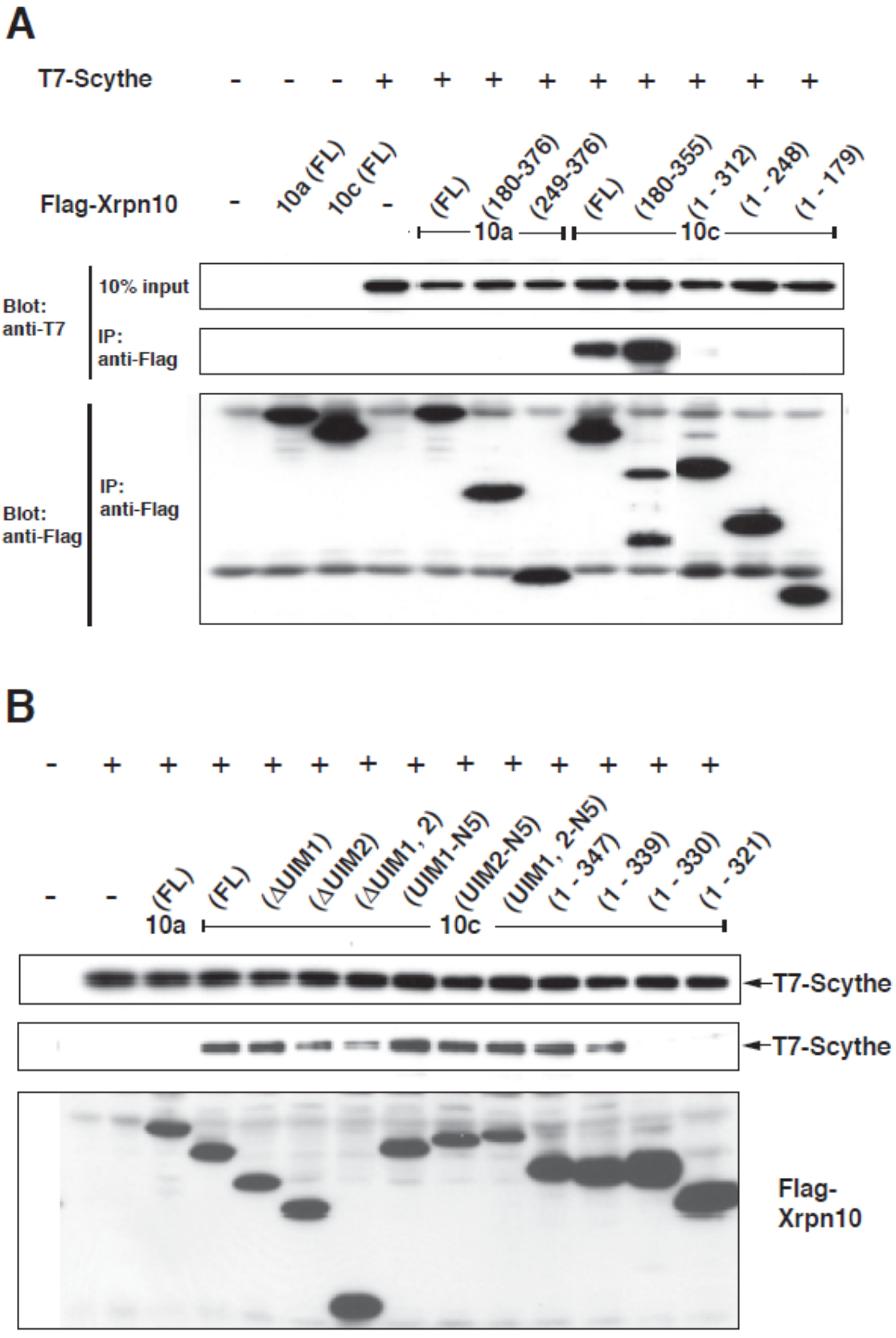
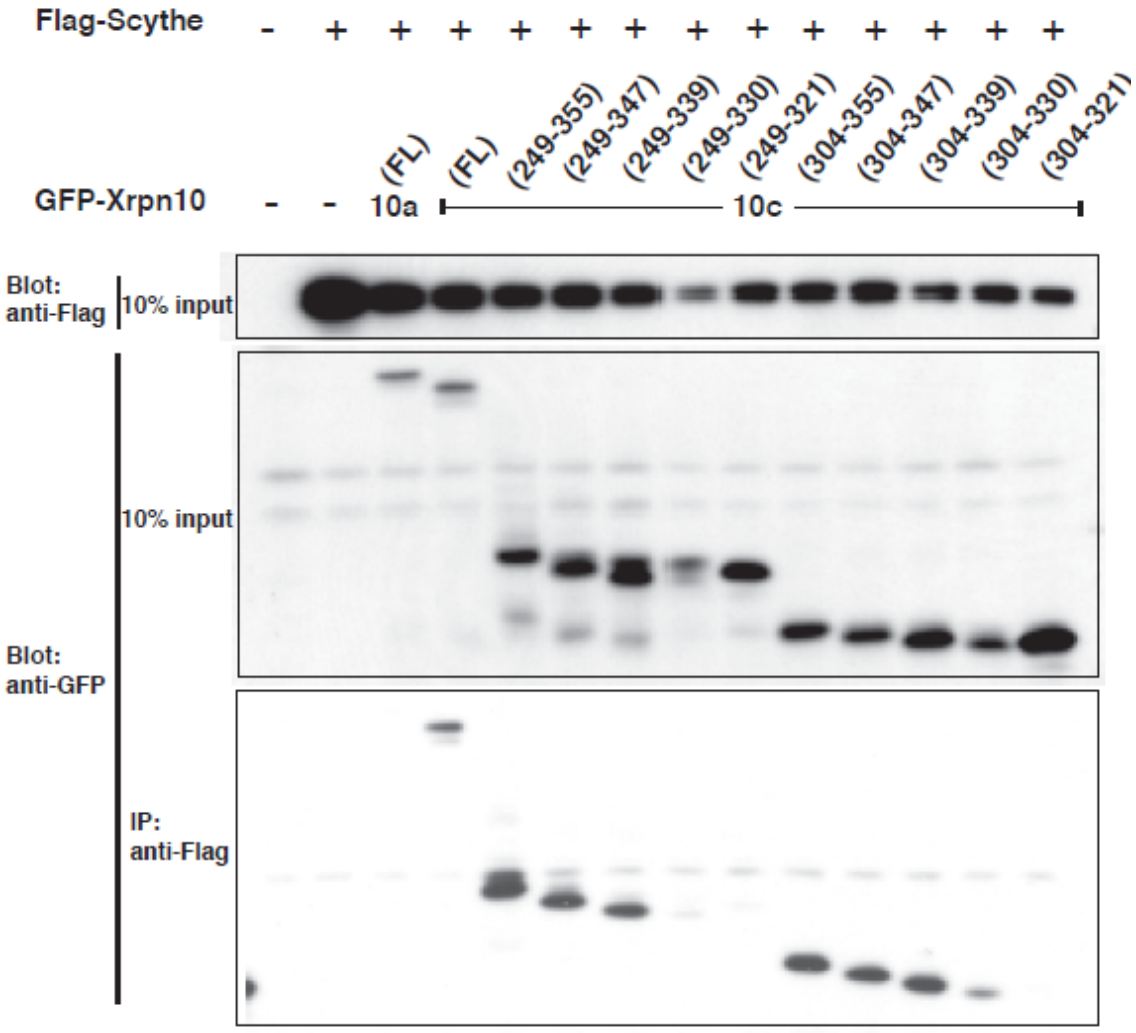

A
T7-Scythe - + + +
Flag-Xrpn10c - - + -
Flag-Xrpn10a - - - +
Blot: anti-T7
10% input
IP:anti-Flag
Scythe
*
Scythe
Blot: anti-Flag
10% input
IP:anti-Flag
Xrpn10a
Xrpn10c
Xrpn10a
Xrpn10c
B
Flag-Xrpn10c - + -
Flag-Xrpn10a - - +
Blot: anti-Flag
10% input
IP:anti-Rpt6
Xrpn10a
Xrpn10c
Xrpn10a
Xrpn10c
Blot: anti-Scythe
10% input
IP:anti-Rpt6
IP:anti-20S
Scythe
Scythe
Scythe
Blot: anti-Rpt6
IP:anti-20S
Rpt6

**Fig. 11. Xrpn10c interacts with Scythe via Rpn10c-specific region.**

(A) T7-tagged Scythe and various deletion mutants of Flag-tagged Xrpn10 were expressed in COS7 cells as indicated. Cell extracts were immunoprecipitated with anti-Flag M2 agarose beads, and the precipitates were immunoblotted with anti-T7 and anti-Flag antibodies. FL represents the full-length form of either Xrpn10a or Xrpn10c. (B) The ubiquitin-interacting motifs (UIM1 and UIM2) of Xrpn10c are dispensable for Scythe interaction. ΔUIM1 indicates specific elimination of amino acid residues 196-241, and ΔUIM2 indicates specific elimination of amino acid residues 263-307. UIM1-N5 and UIM2-N5 indicate site-directed substitution of the core sequences of UIM1 and UIM2 to successive five Asn residues (LALAL for UIM1 and IAYAM for UIM2 to NNNNN, respectively). The results of the experiment on the effects of continuous C-terminal deletion of Xrpn10c (1-347, -339, -330, -321) indicated that Xrpn10c (1-339) is sufficient for Scythe binding. (C) Flag-tagged Scythe and various regions of GFP-tagged Xrpn10c were co-expressed in COS7 cells as indicated. The cell extracts were immunoprecipitated with anti-Flag M2 agarose beads, and the precipitates were immunoblotted with anti-GFP antibody. (D) Schematic representation of various deletion mutants of Xrpn10c. The 10c-box is indicated by the open box. Successful Scythe interactions with Xrpn10 fragments are represented as (+) and failures are represented as (-).


A
T7-Scythe
Flag-Xrpn10
10a (FL)
10c (FL)
(FL)
(180-376)
(249-376)
(FL)
(180-355)
(1 - 312)
(1 - 248)
(1 - 179)
10a
10c
Blot: anti-T7
10% input
IP: anti-Flag
Blot: anti-Flag
IP: anti-Flag
B
(FL)
(FL)
(ΔUIM1)
(ΔUIM2)
(ΔUIM1, 2)
(UIM1-N5)
(UIM2-N5)
(UIM1, 2-N5)
(1 - 347)
(1 - 339)
(1 - 330)
(1 - 321)
10a
10c
T7-Scythe
T7-Scythe
Flag-Xrpn10

C


Flag-Scythe
- + + + + + + + + + + + + +
(FL)
(FL)
(249-355)
(249-347)
(249-339)
(249-330)
(249-321)
(304-355)
(304-347)
(304-339)
(304-330)
(304-321)
GFP-Xrpn10
- - 10a
10c
Blot:
anti-Flag
10% input
10% input
Blot:
anti-GFP
IP:
anti-Flag

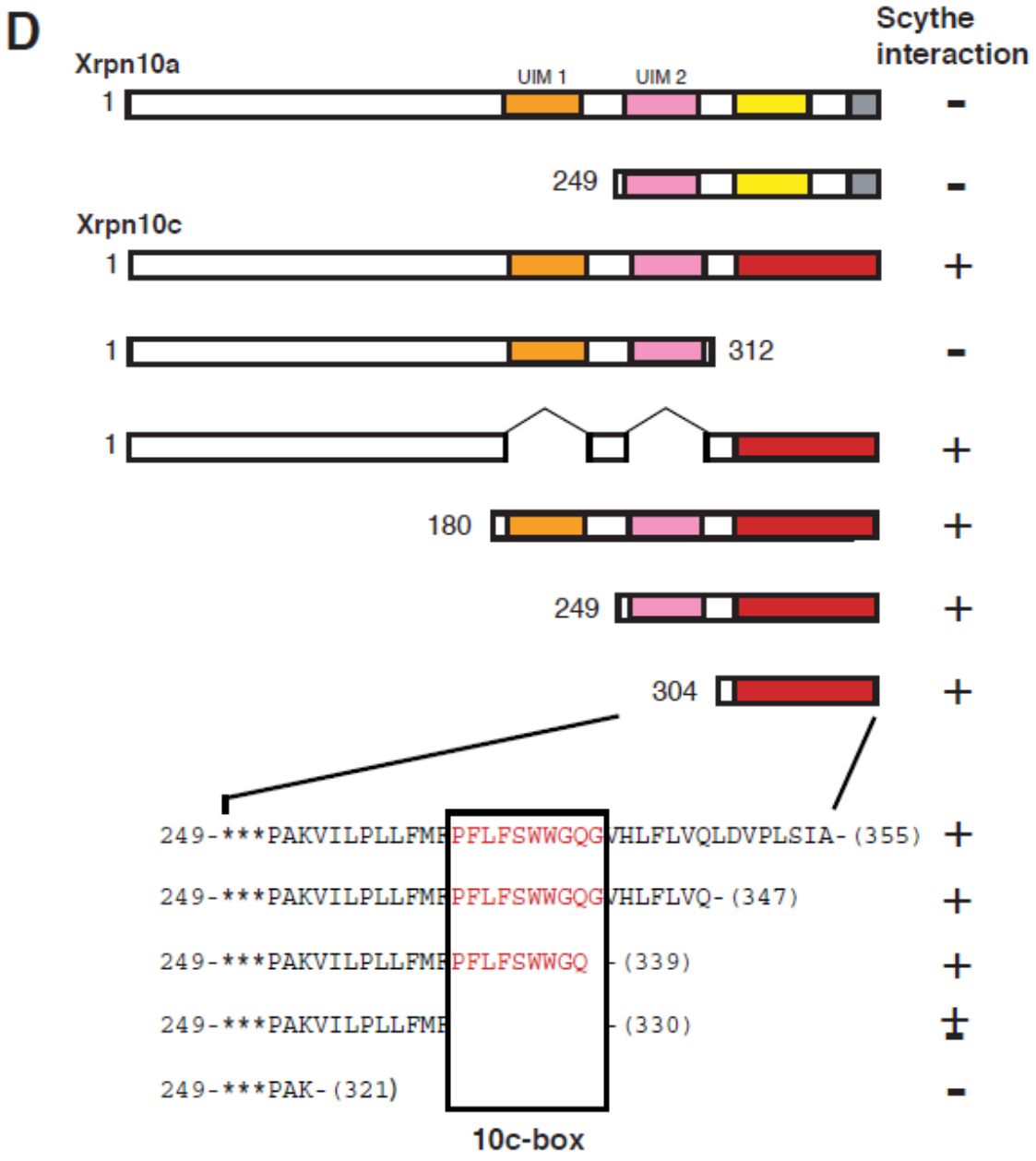

D
Scythe interaction
Xrpn10a
UIM 1
UIM 2
1
-
249
-
Xrpn10c
1
+
1
312
-
1
+
180
+
249
+
304
+
249-***PAKVILPLLFMRPFLFSWWGQGVHLFLVQLDVPLSIA-(355)
+
249-***PAKVILPLLFMRPFLFSWWGQGVHLFLVQ-(347)
+
249-***PAKVILPLLFMRPFLFSWWGQ-(339)
+
249-***PAKVILPLLFMR-(330)
±
249-***PAK-(321)
-
10c-box

**Fig. 12. Xrpn10c interacts with two independent N-terminal domains of Scythe.** (A) Flag-tagged Xrpn10c and various deletion constructs of T7-tagged Scythe were expressed in COS7 cells as indicated. Cell extracts were immunoprecipitated with anti-Flag M2 agarose beads, and the precipitates were immunoblotted with anti-T7 and anti-Flag antibodies. Note that open arrows denote the mutant Scythe signal that did not co-precipitate with Flag-Xrpn10c. HC and LC represent protein bands of heavy- and light-chain of IgG, respectively. (B) Schematic representation of various deletion mutants of Scythe. Note that there are two independent Xrpn10c-binding domains in the N-terminus of Scythe (Domain I and Domain II). Xrpn10c-binding with Scythe fragments is represented as (+) and its failure is represented as (-) on the right.

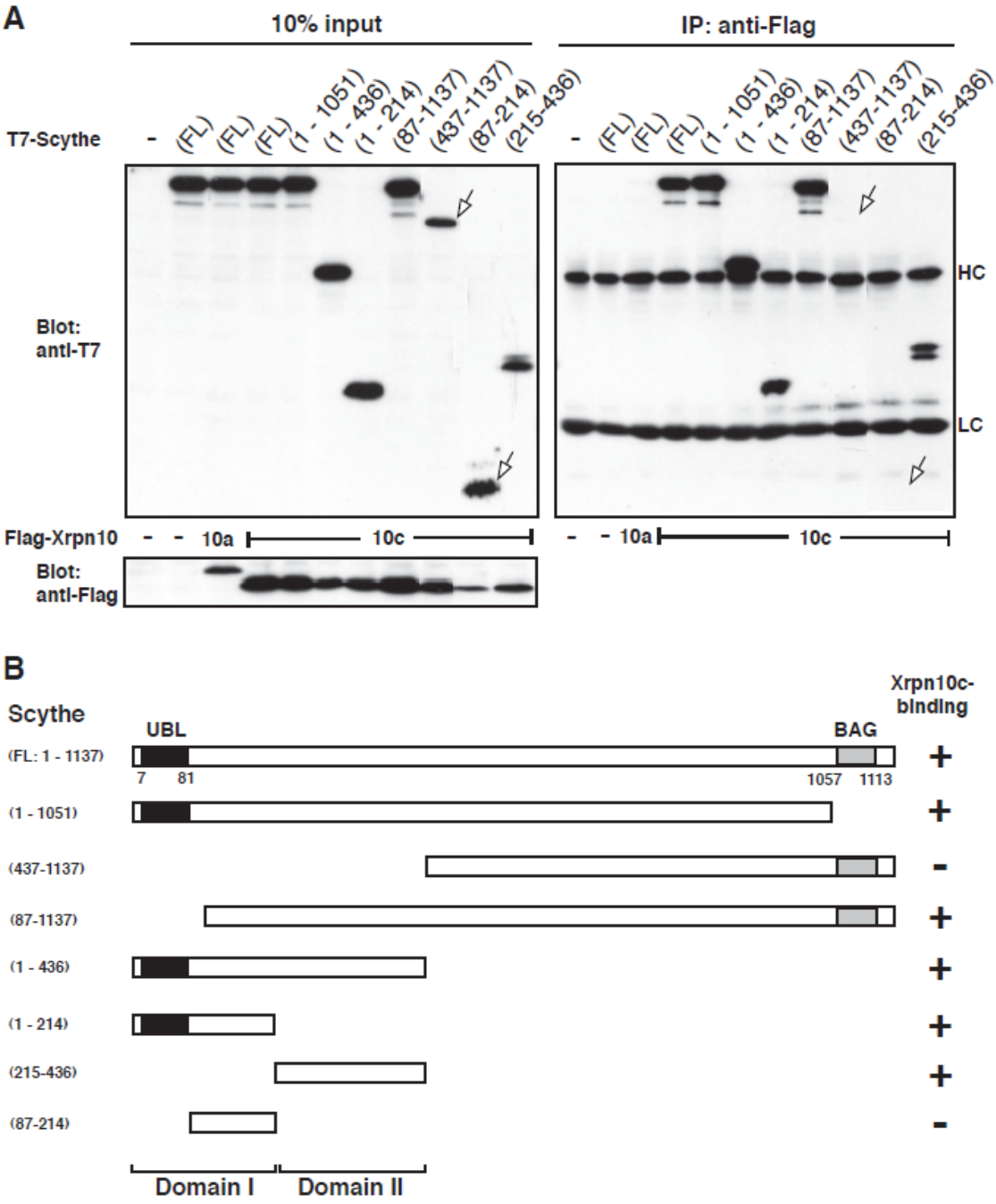

A
10% input
IP: anti-Flag
T7-Scythe
-
(FL)
(FL)
(FL)
(1 - 1051)
(1 - 436)
(1 - 214)
(87-1137)
(437-1137)
(87-214)
(215-436)
HC
LC
Blot:
anti-T7
Flag-Xrpn10
10a
10c
Blot:
anti-Flag
B
Scythe
Xrpn10c-
binding
UBL
BAG
(FL: 1 - 1137)
7
81
1057
1113
(1 - 1051)
(437-1137)
(87-1137)
(1 - 436)
(1 - 214)
(215-436)
(87-214)
Domain I
Domain II

**Fig. 13. Xrpn10c or its 10c-box fragment directly binds with the N-terminal fragments of Scythe *in vitro*.**

(A) Bacterially-expressed GST-fusion proteins as indicated were purified and mixed with bacterially-expressed non-tagged Xrpn10a or Xrpn10c, and the mixture was subjected to an *in vitro* GST pull-down assay with glutathione-Sepharose beads. Precipitants were immunoblotted with an anti-Xrpn10 antibody that recognizes the N-terminal region of both Xrpn10a and Xrpn10c. GST that was fused with the N-terminal 435-a. a. fragment of Scythe and GST that fused with the C-terminal 313-a. a. fragment of Scythe were designated as GST-Scythe (N435) and GST-Scythe (C313), respectively. GST-XHR23B was used as a positive control. (B, C) Bacterially-expressed GST-fusion proteins as indicated were mixed with bacterially-expressed non-tagged Scythe Domain I (B) or Domain II (C), and the mixture was subjected to an *in vitro* GST pull-down assay. Precipitants were immunoblotted with anti-Scythe antibodies. GST that was fused with the 10c-box fragment (9 a. a.) was designated as GST-10c-box. Note that the molecular masses of Scythe Domain I and Domain II correspond to 32 kDa and 36 kDa, respectively. Asterisks indicate partial truncated forms of Xrpn10c.

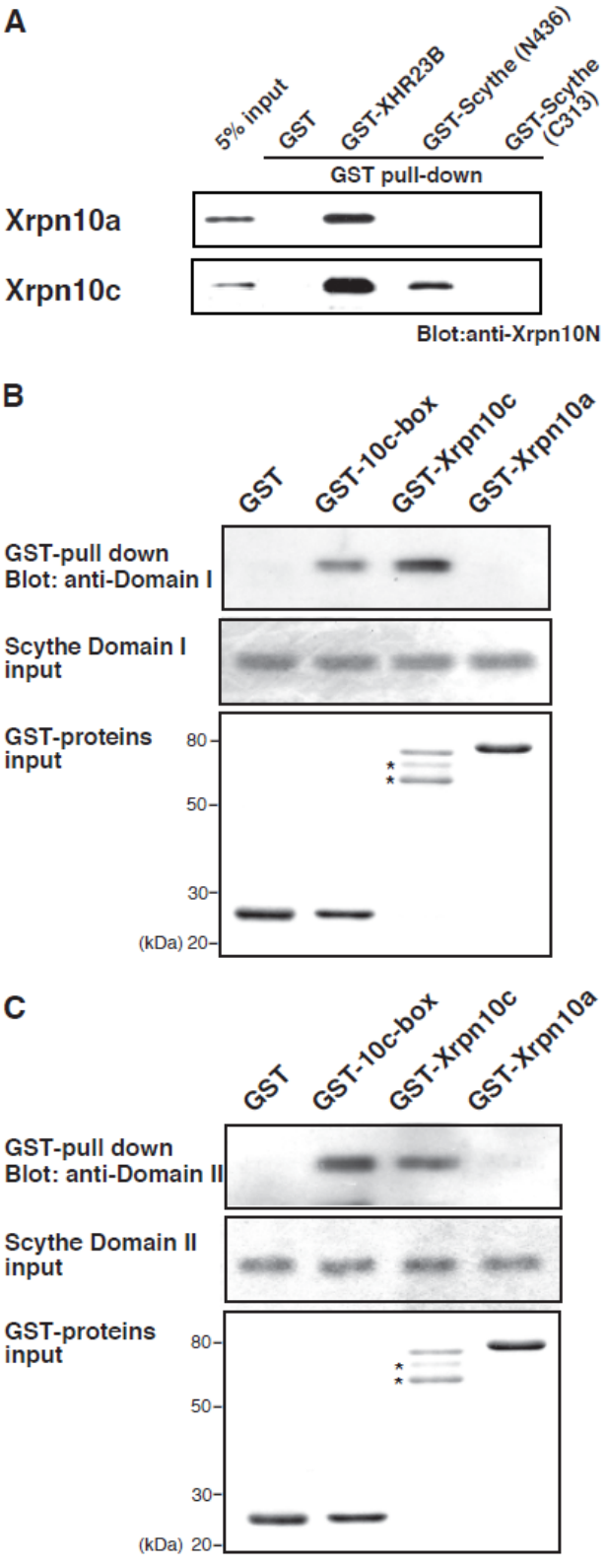

A
5% input
GST
GST-XHR23B
GST-Scythe (N436)
GST-Scythe (C313)
GST pull-down
Xrpn10a
Xrpn10c
Blot:anti-Xrpn10N
B
GST
GST-10c-box
GST-Xrpn10c
GST-Xrpn10a
GST-pull down
Blot: anti-Domain I
Scythe Domain I
input
GST-proteins
input
80
50
30
(kDa) 20
C
GST
GST-10c-box
GST-Xrpn10c
GST-Xrpn10a
GST-pull down
Blot: anti-Domain II
Scythe Domain II
input
GST-proteins
input
80
50
30
(kDa) 20

**Fig. 14. Tandem ubiquitin homology domains contribute to Xrpn10c binding of Scythe.**

(A) Multiple alignments of ubiquitin homology domains of Scythe, UBL1 (7-81), UBL2 (257-323) and ubiquitin. Amino acid residues that are conserved in all three sequences are shown by closed boxes, and those that are conserved in two sequences are shown by shaded boxes. (B) Flag-tagged Xrpn10c and various deletion constructs of T7-tagged Scythe Domain II were expressed in COS7 cells as indicated. Cell extracts were immunoprecipitated with anti-Flag M2 agarose beads and subsequently blotted with anti-T7 antibody. (C) Schematic representation of deletion constructs of Scythe Domain I and II. UBL1 and UBL2 are indicated by closed boxes. Note that ubiquitin homology region of Domain I and II are required but not sufficient for Xrpn10c-binding.

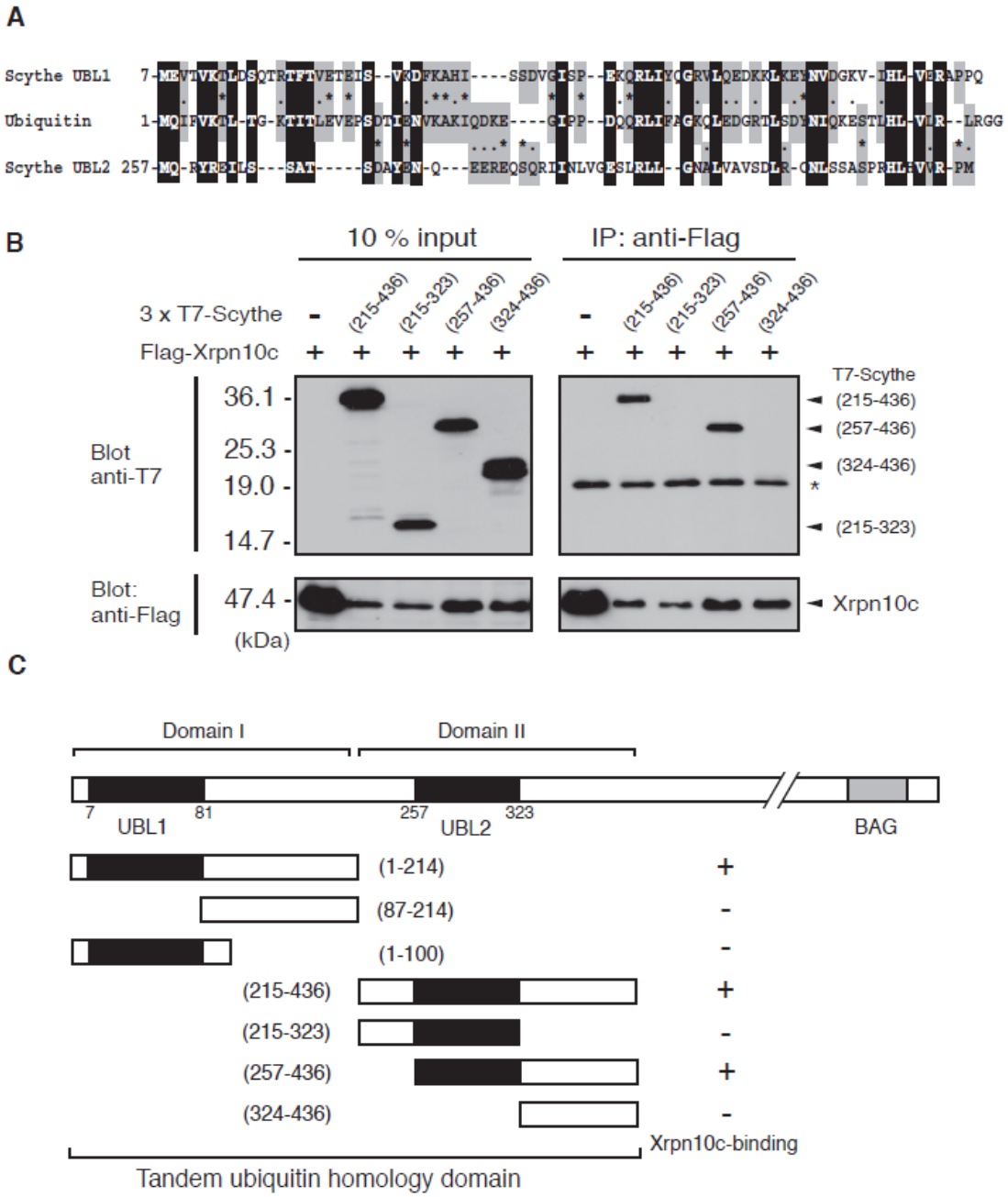
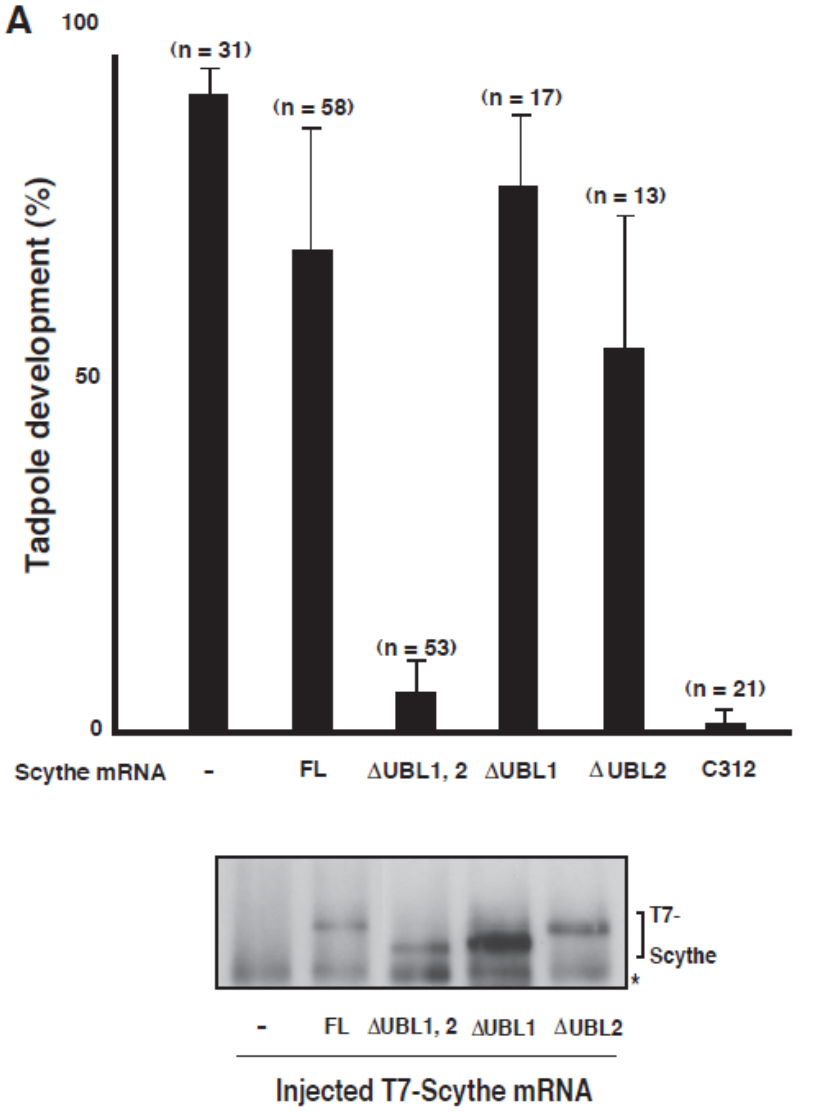

A
Scythe UBL1
Ubiquitin
Scythe UBL2
B
10 % input
IP: anti-Flag
3 x T7-Scythe
Flag-Xrpn10c
(215-436)
(215-323)
(257-436)
(324-436)
Blot
anti-T7
Blot:
anti-Flag
36.1
25.3
19.0
14.7
47.4
(kDa)
T7-Scythe
Xrpn10c
C
Domain I
Domain II
UBL1
UBL2
BAG
7
81
257
323
(1-214)
(87-214)
(1-100)
Xrpn10c-binding
Tandem ubiquitin homology domain

**Fig. 15. UBL1 and UBL2 domains of Scythe are redundantly required for the appropriate development of *Xenopus* embryos.**

Synthetic mRNA encoding Flag-tagged Scythe and its variant proteins were microinjected into *Xenopus* embryos. (A) Ectopic expression of T7-tagged C-terminal 312 a. a. fragment of Scythe (designated as C312) as a positive control resulted in complete elimination of normal tadpole development of injected *Xenopus* embryos, while that of full-length Scythe (FL) (as a negative control) did not influence normal development. Neither the expression of ΔUBL1 nor that of the ΔUBL2 form of Scythe caused detectable developmental abnormality. In contrast, the expression of Scythe protein lacking both UBL1 and UBL2 (ΔUBL1, 2) greatly reduced the rate of tadpole development. Data shown in A represent the mean ±SD of the indicated number of embryos (upper panel). Extracts from an each embryo were probed with anti-T7 antibody to verify the expression of each form of Scythe (lower panel). (B) Schematic representation of Scythe and its mutant derivatives that were expressed in *Xenopus* embryos. UBL and BAG domains are indicated by closed and shaded boxes, respectively.


A
100
50
0
Tadpole development (%)
(n = 31)
(n = 58)
(n = 53)
(n = 17)
(n = 13)
(n = 21)
Scythe mRNA
-
FL
ΔUBL1, 2
ΔUBL1
ΔUBL2
C312
T7-
Scythe
*
-
FL
ΔUBL1, 2
ΔUBL1
ΔUBL2
Injected T7-Scythe mRNA
B
UBL1
UBL2
BAG
Scythe FL
7
81
258
324
1057
1113
ΔUBL1, 2
ΔUBL1
ΔUBL2
C312

# General Discussion

It has been well recognized that the 26S proteasome system plays an important role in the degradation of the majority of intracellular proteins in eukaryotic cells (Collins and Goldberg, 2017). The function of each subunit of the 26S proteasome, however, has not been fully characterized. Through my studies, I focused on Rpn10, a regulatory subunit of the 26S proteasome, to shed the light on a molecular function of Rpn10 in vertebrates.

I clarified the genomic organization of Rpn10 gene in lower vertebrates *Oryzias latipes* (Japanese rice fish) and *Xenopus laevis* (African clawed frog), and provided evidence for the generation of Rpn10 isoforms in an alternative splicing-manner among vertebrates. Although this was not discussed in the Chapter 1 because my primary object of the study was to show the genomic organizations of the vertebrate Rpn10, I also revealed that the Rpn10 gene of *Ciona intestinalis* (ascidian) is comprised of 7 exons, and retains the exon-intron junctions which potentially generate Rpn10 isoforms corresponding to mouse Rpn10c and Rpn10d (Fig. 16). My study on the genomic organization of Rpn10 gene was mostly achieved by complicated sequential PCR strategy in combination with degenerate PCR, nested PCR, and conventional PCR techniques. On the other hand, the recent advances of high-throughput sequencing technologies and public genomic databases have enabled me to examine Rpn10 genes of a wide variety of organisms. Given this situation, I searched for genomic structures of Rpn10 of additional organisms in databases to raise the confidence levels of the findings established by my study. I investigated several vertebrates including wolf (*Canis lupus*), chicken (*Gallus gallus*), zebrafish (*Danio rerio*), and several eukaryotic protists (*Tetrahymena thermophila, Paramecium tetraurelia*, and *Trypanosoma brucei*), and plants (*Arabidopsis thaliana* and *Oryza*

*sativ*e) at the NCBI Gene Database (https://www.ncbi.nlm.nih.gov/gene/) or elsewhere. This investigation indicated that the positions of the exon-intron junctions and the length of each exon are similar between vertebrates and plants but different in eukaryotic protists as in yeast, nemotode, and fruit fly. This is consistent with the findings brought up from my study discussed in the Chapter 1 in details, although sequencing analysis is essential to completely validate the observations from database information. Collectively, these findings indicate that Rpn10 molecule might have acquired complexities generated by alternative splicing at its C-terminal stretch through evolution to meet the requirement for expanded diversity of ubiquitin-mediated protein degradation.

I also elucidated a specific function of one of the Rpn10 isoforms, Rpn10c for the first time. I revealed that Xrpn10c is a Scythe-binding protein and Scythe has tandem UBL domains that are essential for the interaction with Xrpn10c. It was also indicated by my study that the tandem UBL domains of Scythe are redundantly implicated in the embryogenesis during *Xenopus* development. These findings clearly show the significance of Rpn10c and provide the first evidence that shows N-terminal tandem domains of Scythe act as essential regions linking the ubiquitin/proteasome machinery to the control of *Xenopus* embryonic development. Based on my studies, Minami et al. searched for a novel Scythe-binding protein from *Xenopus* embryo and identified XEF1AO (Minami et al., 2007), which has been reported as a potential apoptotic inducer in vertebrates (Kato et al., 1997; Ruest et al., 2002), and showed that Scythe regulates apoptosis through modulation of ubiquitin-mediated degradation of XEF1AO. This is a consistent finding with my study and would suggest a potential role

of Xrpn10c-specific proteasome in the regulation of apoptosis during *Xenopus* embryogenesis, although this might still be a preliminary assumption at this stage.

As mentioned in the General Introduction, the ubiquitin proteasome pathway is conserved among eukaryotes. It was well studied and shown that most of deletion or nonfunctional mutants of 26S proteasome subunits are lethal in yeast (Tanaka, 2009), suggesting the 26S proteasome is indispensable for cell survival in eukaryotes. Although knockout studies of the 26S proteasome in vertebrates are few, it was reported that Psmc3 (Rpn5) and Psmc4 (Rpn3)-deficient mice are embryonic lethal, suggesting that the 26S proteasome plays an essential role in development and embryogenesis in mammals (Sakao et al., 2000). It was also shown that neuron-specific Psmc1 (Rpn2)-knockout mice exhibit 26S proteasomal dysfunction and neurodegeneration, indicating that the 26S proteasome is essential for normal neurodevelopment as well (Bedford et al., 2008). In addition, although yeast Rpn10 mutants are vulnerable to stress but not lethal (van Nocker et al., 1996b; Wilkinson et al., 2000), it has been reported that Rpn10 KO mice shows an embryonic lethality (Hamazaki et al., 2007). This is a consist observation with my results which indicated that Rpn10 plays an important role in the embryogenesis in *Xenopus*. It was also shown by Hamazaki et al. that mice expressing the N-terminal portion of Rpn10, which contains VWA domain but lacked UIMs exhibited embryonic lethality. These observations suggest that Rpn10 is indispensable for survival and development at least in mice, and its UIMs have distinct roles in mammals from yeast, as was indicated by my studies. However, Hamazaki et al. also showed that Rpn10a knock-in (KI) mice, which exclusively express Rpn10a (i.e. the constitutive type of Rpn10) and do not express other Rpn10 isoforms, are born normally at Mendelian frequency, suggesting that Rpn10b-to-Rpn10e isoforms are

dispensable for mouse embryogenesis. This observation could bring up a question if Rpn10 isoforms are important in frogs but this is not the case for mice, even though the experimental settings were completely different from those in my study. This potential difference between mice and frogs can be explained simply by species difference or by compensatory mechanisms in the case of Rpn10 KI mice. Although they showed that some of the proteasome-related protein levels were comparable between wild-type and Rpn10a KI mice, it is likely that the other proteins (e.g. substrate proteins, regulatory proteins) which they did not examine are up- or down-regulated to adapt to the environment and maintain protein homeostasis as Rpn10a KI mice lack Rpn10b-to-Rpn10e from the beginning of the life. In contrast, in the case of my study, aberrant forms of Scythe that lacked both UBLs and were exogenously expressed after fertilization and in the middle of embryogenesis, the timing of which Xrpn10c plays a crucial role in, caused the dysregulation of protein degradation by the Xrpn10c-specific 26S proteasome. To address possible functions of Rpn10 splicing variants in mouse embryogenesis, it would be interesting to examine the knockdown of each Rpn10 isoform mRNA during development stages. This is expected to be conducted in the future studies.

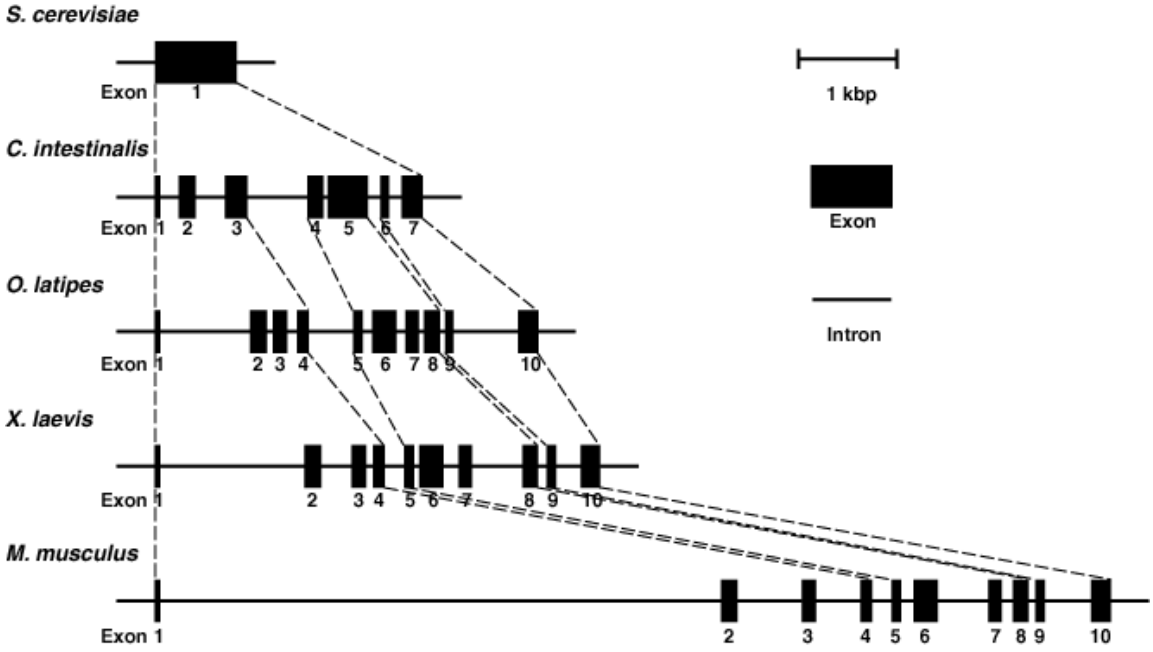
Physiological roles of Scythe have been more and more elucidated since I published my studies. Scythe is currently considered to be essential for the quality control of protein biogenesis as a chaperone molecule in association with CHIP and Hsp70 as well as play important roles in antigen presentation, the T-cell response, and apoptosis (Binici and Koch, 2014; Kawahara et al., 2013). Although I focused my research on the role of Rpn10 isoforms in the embryonic development because they were originally subcloned from embryonic cDNAs, it is possible that at least some of

the Rpn10 isoforms are expressed in adults as well and play some roles in the regulation of various physiological functions in which Scythe is implicated. In addition, it is likely that there are other Rpn10c-binding proteins than Scythe. These questions are expected to be addressed in future studies.

In conclusion, my first study suggests that vertebrate Rpn10 molecule has acquired the unique diversity which is not observed in other proteasome subunits, presumably to meet the requirement for expanded role of the 26S proteasome in protein degradation through evolution. In addition, my second study provides clear evidence linking the Rpn10c to the control of embryonic development. As embryogenesis is the active process that generates new cells and tissues within a short period of time, it may require another protein degradation mechanism by Rpn10 isoforms to enhance the activity and substrate selectivity of the 26S proteasome. It is highly valuable that my studies shed the light on a novel substrate recognition mechanism of the 26S proteasome by an Rpn10 splicing variant and its role in normal embryonic development.

**Fig. 16. Physical maps of genomic organization of yeast, ascidian, fish, frog, and mouse Rpn10 genes.**

The scale shows the length of 1 kbp. Exons are indicated by solid boxes and numbered from 1 to 10. Yeast, *Saccharomyces cereviciae*; Ascidian, *Ciona intestinalis*; Japanese rice fish, *Oryzias latipes*; African clawed frog, *Xenopus laevis*; and mouse, *Mus musculus*.


S. cerevisiae
Exon 1
C. intestinalis
Exon 1 2 3 4 5 6 7
O. latipes
Exon 1 2 3 4 5 6 7 8 9 10
X. laevis
Exon 1 2 3 4 5 6 7 8 9 10
M. musculus
Exon 1 2 3 4 5 6 7 8 9 10
1 kbp
Exon
Intron

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