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Differential expression pattern of UBX family genes in *Caenorhabditis elegans*

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Abstract

UBX (ubiquitin regulatory X)-containing proteins belong to an evolutionary conserved protein family and determine the specificity of p97/VCP/Cdc48p function by binding as its adaptors. *Caenorhabditis elegans* was found to possess six UBX-containing proteins, named UBXN-1 to -6. However, no general or specific function of them has been revealed. During the course of understanding not only their function but also specified function of p97, we investigated spatial and temporal expression patterns of six *ubxn* genes in this study. Transcript analyses showed that the expression pattern of each *ubxn* gene was different throughout worm's development and may show potential developmental dynamics in their function, especially *ubxn-5* was expressed specifically in the spermatogenic germline, suggesting a crucial role in spermatogenesis. In addition, as *ubxn-4* expression was induced by ER stress, it would function as an ERAD factor in *C. elegans*. *In vivo* expression analysis by using GFP translational fusion constructs revealed that six *ubxn* genes show distinct expression patterns. These results altogether demonstrate that the expression of all six *ubxn* genes of *C. elegans* is differently regulated.

Keywords: AAA ATPase; *C. elegans*; Chaperone; ERAD; Gene expression; p97/VCP/Cdc48; Spermatogenesis; UBX

The evolutionary conserved AAA ATPase p97 (also called VCP in higher eukaryotes and Cdc48p in yeast) is involved in a variety of diverse cellular processes, including ERAD (endoplasmic reticulum-associated protein degradation), homotypic membrane fusion, transcriptional regulation, cell-cycle regulation, spindle disassembly, and other protein quality control activities [1–3]. The common function of p97 is believed to be the ATP-dependent protein conformational change; unfolding of proteins and disassembly of protein complexes [4]. Specificity of different p97-dependent processes is mainly determined by differential binding of distinct cofactors/adaptor proteins [3]. For example, when bound to the Ufd1/Npl4 complex, p97 functions in ubiquitin-dependent protein degradation pathways, including activation of membrane-tethered SPT23 transcription factor, and retrotranslocation in ERAD [5,6], whereas when bound to p47 or p37, which is a member of UBX (ubiquitin regulatory X)-containing protein family, it functions solely in membrane fusion [7–9].

UBX-containing proteins belong to an evolutionarily conserved protein family defined by the presence of the UBX domain, which is composed of 80 amino acids [10]. Structural studies of the UBX domain of FAF-1 and p47 have shown that the UBX domain adopts a characteristic ubiquitin fold and that this domain is a p97-binding region [10,11]. In *Saccharomyces cerevisiae* genome, there are seven UBX-containing proteins including the putative yeast orthologue of p47, Sph1. A systematic analysis of the yeast UBX-containing proteins revealed that all of them bind to Cdc48 (p97) via the UBX domain [12]. It has been demonstrated that yeast Ubx2 recruits Cdc48 to ubiquitin ligases and thus is a positive-regulator of ERAD [12–14]. Recently, human UBXD2, named erasin, and human FAF-1 have been shown to be involved in protein

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degradation in the ubiquitin–proteasome pathway [15,16]. In contrast, an *Arabidopsis* UBX-containing protein, PUX1, regulates p97 function by inhibiting its ATPase activity and by promoting the disassembly of the active p97 hexamer [17].

Caenorhabditis elegans uniquely possesses two p97 homologues, named CDC-48.1 (C06A1.1) and CDC-48.2 (C41C4.8) [18]. We have reported that *C. elegans* two p97 homologues are essential and functionally redundant and that their expression is differently regulated [18,19]. Furthermore, we have revealed that *C. elegans* p97 is involved in ERAD, sex determination, organ development, meiotic processes and so on [Sasagawa et al., manuscript in preparation]. As described above, differential binding of adaptor proteins to p97 plays a crucial role in determination of specificity of p97 functions. Therefore, we have initiated the systematic analysis of *C. elegans* UBX-containing proteins to elucidate the global regulation of p97 functions.

In this study, we investigated spatial and temporal expression patterns of six *ubxn* genes in *C. elegans*. We also analyzed their response to various stress treatments.

Materials and methods

Strains of C. elegans and culture condition. Wild-type Bristol N2 strain was cultured at 20 °C as described by Brenner [20]. The following temperature-sensitive mutants were also used: *glp-4*(*bn2*)*I*, *fem-3*(*e2006*)*IV*, and *fem-3*(*q20*)*IV*. These strains were maintained at 15 °C and shifted to 25 °C for analysis. For stress treatment, worms were treated with heat-, cold-, starvation-, oxidative-, and ER-stresses as described previously [19].

RNA preparation and determination of the trans-splicing acceptor site. Total RNA was isolated as described previously [19]. RT-PCR was performed with SuperScript III RNaseH$^-$ reverse transcriptase (Invitrogen) and TaKaRa Ex Taq (TaKaRa). First-strand cDNA was synthesized from 1 μg of total RNA with random hexamers. After mRNA degradation, two-step nested PCR was carried out. First round PCR was done with a set of primers of SL1 sequence and each gene-specific primer (Table 1, First PCR). The gene-specific primers for first round PCR were substituted with second PCR primers for the second round PCR (Table 1, Second PCR). The amplified DNAs were cloned into T-vector (Novagen) and sequenced using an ABI 310 autosequencer (Applied Biosystems).

Northern blot analysis and real-time PCR. Northern blot analysis was performed as described previously [21]. Each probe specific to six *ubxn* genes was prepared and detected with PCR DIG Probe Synthesis Kit and DIG Luminescent Detection Kit (Roche Diagnostics). Hybridization with *act-1* mRNA was used as a loading control. Real-time PCR was carried out using SYBR PrimeScript RT-PCR Kit (TaKaRa) and ABI 7500 system (Applied Biosystems). First-strand cDNA was synthesized from 2 μg of total RNA with random hexamers. For PCR, primers used were listed in Table 1 and cycling condition was: 15 s at 95 °C; 40 cycles of 5 s at 95 °C and 34 s at 60 °C. The 18S rRNA was simultaneously quantified as an internal control.

GFP fusion constructs and microscopic observation. Upstream regions encompassing regulatory regions of six *ubxn* genes were amplified with primers (Table 1). PCR products were cloned into the plasmid pPD95.77 to create a translational fusion to green fluorescent protein (GFP). Establishment of transgenic worms was carried out as described previously [19]. At least three independent transgenic lines for each GFP construct were isolated and analyzed. Worms were mounted on 3% agarose pads for fluorescence microscopy on an Olympus BX51 microscope equipped with a CCD camera. Adobe Photoshop 5.0 was used for output of images.

Table 1
Oligonucleotides used in this study

Probe synthesis for Northern blot analysis and real-time PCR		
ubxn-1	1	AAGCTTCTCGCCAACGA
	2	TTCACGATCTCTGGCGG
ubxn-2	1	CACTTGTTCAACAATATCC
	2	GTCGTTGGCTCATTCATA
ubxn-3	1	ACTGTTTCCACGCTTATTCG
	2	ATACTCAGCCTCCTGCTGA
ubxn-4	1	GTTTGGTGGCAACGTAG
	2	CTGGCAGAATTGGTGTAG
ubxn-5	1	ATGCCAACTAACAATCACGTAC
	2	GGTATCGTACTAGAAGACGG
ubxn-6	1	ATCCGGCGAGTCATCTT
	2	CAACTCGGAAGTGTAGAG
GFP construct		
ubxn-1	3	GCATGCGAAATAATCTGAAAAAGACC
	4	GGATCCATCATCGTTGGCGAGAAGC
ubxn-2	3	CTGCAGATTATTACATCGTGGTTGATT
	4	GTCGACTTCGGCGGCCTGAAGG
ubxn-3	3	GCATGCAAATTGCCGATTTGCCAG
	4	GTCGACTTCTGGCTCGAATCTCGA
ubxn-4	3	GCATGCAGCTGCTTGATTTACGC
	4	GTCGACCGTTGACGCTGGTACTTGG
ubxn-5	3	GCATGCAATAAAGACATGAAAAGGCA
	4	GTCGACAAACTTCTCCTTTTGTACTGTG
ubxn-6	3	GCATGCATGAAGTCGTTCTCATTCTTT
	4	GGATCCACAATACTTGCAGATTGTTTCG
Determination of the trans-splicing acceptor site		
SL1		GGTTTAATTACCCAAGTTTG
First PCR		
ubxn-1	2	TTCACGATCTCTGGCGG
ubxn-2	2	GTCGTTGGCTCATTCATA
ubxn-3	5	ATTGATCACTTCTGGCTC
ubxn-4	2	CTGGCAGAATTGGTGTAG
ubxn-5	2	GGTATCGTACTAGAAGACGG
ubxn-6	2	CAACTCGGAAGTGTAGAG
Second PCR		
ubxn-1	5	CTGCAGTGAGAGGCTTG
ubxn-2	5	CAGTATGATCCGGGTTATG
ubxn-3	6	CGAGCTCGACGATGCTC
ubxn-4	5	ATATCAGCAAACTGCTGG
ubxn-5	5	GGTCCTTGTGATACAGTTC
ubxn-6	5	GTGATATTGCGGATGAATG

Results and discussion

All six ubxn genes are expressed in C. elegans

In silico homology search using a UBX domain sequence, which is composed of 80 amino acids [10], we found that *C. elegans* possesses six UBX-containing proteins, named UBXN-1 to -6 (Fig. 1A). Taken together with previously reported information, most of UBX-containing proteins can be grouped into seven evolutionary conserved families that are represented by the human UBXD-1, UBXD-2, UBXD-7, FAF-1, p47, SAKS1 (Y33K), and UBXD-6 (Rep8) [10,15,22]. *C. elegans* UBXN-1, UBXN-3, UBXN-4, and UBXN-6 were found to be classified into SAKS1, FAF-1, UBXD-2, and UBXD-1 groups, respec-