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ATX-3, CDC-48 and UBXN-5: A new trimolecular complex in *Caenorhabditis elegans*

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ABSTRACT

Ataxin-3 is the protein involved in Machado–Joseph disease, a neurodegenerative disorder caused by a polyglutamine expansion. Ataxin-3 binds ubiquitylated proteins and acts as a deubiquitylating enzyme *in vitro*. It was previously proposed that ataxin-3, along with the VCP/p97 protein, escorts ubiquitylated substrates for proteasomal degradation, although other players of this escort complex were not identified yet.

In this work, we show that the *Caenorhabditis elegans* ataxin-3 protein (ATX-3) interacts with both VCP/p97 worm homologs, CDC-48.1 and CDC-48.2 and we map the interaction domains. We describe a motility defect in both ATX-3 and CDC-48.1 mutants and, in addition, we identify a new protein interactor, UBXN-5, potentially an adaptor of the CDC-48-ATX-3 escort complex. CDC-48 binds to both ATX-3 and UBXN-5 in a non-competitive manner, suggesting the formation of a trimolecular complex. Both CDC-48 and ATX-3, but not UBXN-5, were able to bind K-48 polyubiquitin chains, the standard signal for proteasomal degradation. Additionally, we describe several common interactors of ATX-3 and UBXN-5, some of which can be *in vivo* targets of this complex.

Introduction

Machado–Joseph disease (MJD) is a late onset neurodegenerative disease caused by a CAG expansion in the *ATXN3* gene, that encodes for ataxin-3 [1]. Ataxin-3 binds polyubiquitylated proteins, exhibits deubiquitylating (DUB) activity *in vitro* and associates with the proteasome, suggesting a role in the ubiquitin–proteasome pathway (UPP) [2,3]. The UPP is responsible for selective proteolysis of proteins, acting both as a regulatory and quality control core of the cell. Target proteins are marked by covalent modification with ubiquitin molecules, by a process known as ubiquitylation [4]. Once the substrates have the correct ubiquitin chain length, escort proteins deliver them to the proteasome for irreversible degradation. One of these escort proteins is the AAA ATPase VCP/p97, the ortholog of yeast CDC-48p [5]. The existence of multiple adaptors and/or cofactors explains the versatility of VCP/p97 and confers functional specificity to this protein. For example, the binding of p47 to VCP/p97 is often associated with homotypic fusion of endoplasmic reticulum (ER) and Golgi membranes, while binding of the Ufd1/Npl4 complex has been associated with ER-associated degradation (ERAD) [6]. The 'Ubiquitin regulatory X' (UBX) domain-containing proteins represent the largest family of VCP/p97 cofactors identified so far [7]. UBX proteins are involved in substrate recruitment to VCP/p97 and it has been shown that they can temporally and spatially regulate its activity [8,9].

VCP/p97 has also been implicated in protein misfolding diseases. VCP/p97 co-localizes with insoluble protein aggregates that are hallmarks of several neurodegenerative disorders, such as the polyglutamine diseases (including MJD), Alzheimer's and Parkinson's diseases [10].

Interestingly, it has been shown that human ataxin-3 associates with VCP/p97 [3] and even though the relevance of ataxin-3/VCP interaction *in vivo* is not fully understood, it has been proposed that this complex is involved in the ERAD pathway, where ataxin-3 seems to promote deubiquitylation of VCP/p97-bound substrates and to modulate their transfer to the proteasome during the process [11,12].

Contrary to other organisms such as human, mouse and yeast, that only possess one VCP/p97, the nematode *C. elegans* has two VCP/p97 homologs named CDC-48.1 and CDC-48.2, that have very distinct expression patterns, suggesting different biological roles [13].

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We have previously showed that the *C. elegans* ataxin-3, ATX-3, is ubiquitously expressed and that it is a DUB enzyme as its human counterpart [14].

In this work, we show that ATX-3 is capable of interacting with the worm CDC-48 proteins and we have identified UBXN-5 as an adaptor of this CDC-48/ATX-3 complex. We describe new ATX-3 and UBXN-5 interactors, some of which are muscle proteins and may be potential *in vivo* targets of this complex. Additionally, we describe a motility defect for *atx-3* and *cdc-48.1* knockout animals.

Materials and methods

Caenorhabditis elegans procedures. Caenorhabditis elegans strains were maintained at 20 °C in NGM plates [15]. Strains used in this work were kindly provided by the CGC. *Atx-3(gk193)*, *cdc-48.1(tm544)* and *cdc-48.2(tm659)* knockout strains were previously described [13,14]. Double *atx-3;cdc-48* mutants were kindly provided by T. Hoppe.

Cloning. The original vectors pAS1–2 and pACT2 (kindly provided by Dr. S. Elledge, Baylor College of Medicine) were converted into yeast two-hybrid (y2h) vectors pAS1g and pACT2g [16]. The *atx-3* cDNA was cloned into the pDONR201 as described [13]. N- and C-terminal portions of *atx-3* were amplified using primers attB1_atx-3 and attB2_atx-3_NT and attB1_atx-3_CT and attB2_atx-3, respectively (Supplementary Table 1). *Ubxn-5* was amplified using primers attB1_ubxn-5 and attB2_ubxn-5 (Supplementary Table 1) and cloned into pDONR207. pDONR201/*cdc-48.1* and pDONR201/*cdc-48.2* were purchased from Open Biosystems. NT e CT portions of the CDC-48 proteins were produced using primers described in Supplementary Table 1. The pDONR clones were transferred into the pAS1g, pACT2g, pDEST-15(GST) and pDEST-17(his) vectors using the Gateway technology (Invitrogen).

Y2h assays. Yeast Y190 was used in all assays. Transformations were performed using the lithium acetate method (Clontech). Single transformants were plated in selective SC medium and tested for auto-activation using different 3-aminotriazole amounts. Double transformations (bait + prey) were performed sequentially. Each bait (or prey) was always co-transformed with empty prey (or bait) plasmid (negative control). The interaction between SNF4 and SNF1, was used as a positive control (kindly provided by Dr. S. Elledge, Baylor College of Medicine). The colony-lift β-galactosidase assays were performed accordingly to regular protocols (Clontech).

Recombinant protein expression and purification. pDEST15/*ubxn-5*, pDEST15/*atx-3* and pDEST17/*cdc-48.1*_NT plasmids were transformed in BL21pLYS. Cultures were grown until OD_{600} of 0.6–0.8 on LB plus ampicillin (50 μg/ml) and glucose (2%). Expression was induced by addition of 1 mM IPTG. Protein was purified using previously described protocols [14,17].

GST-pulldown assays. For pulldowns, 2 μg of GST or GST-tagged proteins were incubated at 4 °C with 20 μl of glutathione–Sepharose beads in 200 μl of binding buffer (PBS 1×, 0.5% Triton X-100, 1 mM PMSF and complete protease inhibitor cocktail (Roche)). At least 500 μg of worm protein extracts were incubated for 3 h at 4 °C. After extensive washing, bound proteins were eluted by boiling in 50 μl of 1× SDS sample buffer and then were analyzed by SDS–PAGE.

For competition assays, His-tagged CDC-48.1_NT was incubated with equimolar amounts of GST-UBXN-5 and with increasing amounts of ATX-3 (2, 4 and 8 μg). After 2–3 h incubation, 30 μl of Ni-beads (Qiagen) were added to the reaction and incubated for an additional hour. After extensive washing, bound proteins were eluted by boiling in 1× SDS sample buffer.

For gels to be analyzed by mass spectrometry, at least 10 μg of recombinant protein and 2 mg of worm extracts were used for pulldowns.

Mass spectrometry. Following affinity purification, samples were run in a regular SDS–Page gel and stained with silver nitrate. We then excised eight protein bands from both GST and GST-ATX-3 lanes. Gel pieces were cut in small pieces using a scalpel. Samples were dehydrated, trypsinized and peptides were analyzed using a nanoflow LC coupled to a QSTAR Pulsar i ESI-hybrid Q-TOF tandem mass spectrometer (Applied Biosystems/MDS Sciex). MS/MS spectra generated were analyzed using Mascot software using the Wormbase wormprep 178 as the reference database (detailed protocol in Supplementary Material).

Motility assays. Synchronized young adults were placed in the middle of a 1 cm circle and after 1 min, animals inside the circle were scored as uncoordinated [18]. More than 50 animals were scored per strain and at least three independent replicates were performed.

Results and discussion

Finding ATX-3 interactors

In order to identify new partners of ATX-3, we have performed an experiment in which GST-tagged ATX-3 was incubated with *C. elegans* protein extracts from a mixed population, to ensure that the largest possible number of worm proteins were present in the sample. We observed a protein band selectively enriched in the ATX-3 pulldown, migrating around 90 kDa, absent from the GST lane (Fig. 1A). MS identified the proteins from the band as being the two *C. elegans* VCP/p97 homologs, CDC-48.1 and CDC-48.2. In addition, and distributed throughout the other bands, we found several muscle-related proteins such as titin, dystrophin, myosin-like protein (CE08251), myotactin (LET-805) and centromere-associated protein E-like (CE40161) (Supplementary Table 2).

ATX-3 and CDC-48 interaction is conserved in C. elegans

To further confirm these results, we performed an independent y2h assay, which proved that indeed ATX-3 directly interacts with CDC-48.1 and CDC-48.2 (Fig. 1B). To map the interaction region, we used different ATX-3 constructs consisting of the Josephin (ATX-3_J1, 1–211 amino acids) or the C-terminal domains (ATX-3_CT, 211–317 amino acids). The ATX-3 protein binds to the CDC-48 proteins through its CT domain (Fig. 1C), similarly to its human counterpart [19].

ATX-3 and CDC-48.1 mutants have a motility defect

To explore the biological significance of this interaction, we examined the phenotype of ATX-3 and CDC-48 mutant animals. As several proteins identified as ATX-3 partners were muscle-related proteins, and since CDC-48/p97 is involved in myosin assembly and myofibril organization, both in *C. elegans* and humans [20], we used a motility assay to score their phenotype (Fig. 2A).

At 20 °C, the *atx-3* and *cdc-48.2* knockouts presented uncoordination levels similar to wild-type. *Cdc-48.1* mutants had a slight increase in uncoordination at this temperature ($p = 0.016$) (Fig. 2B). Surprisingly, at 25 °C, the *atx-3* and *cdc-48.1* mutants displayed 60% and 45% of uncoordination, respectively, while *cdc-48.2* animals behave similarly to N2 (Fig. 2C). The observed phenotypes for *atx-3* and *cdc-48.1* were temperature-dependent, since the *atx-3* phenotype is only manifested at 25 °C while the *cdc-48.1* motility phenotype significantly increased with the temperature. This can indicate that at 25 °C these two proteins are more required, probably to exert a role in the UPP, known to be more active at higher temperatures.