

RTCB-1 Mediates Neuroprotection via XBP-1 mRNA Splicing in the Unfolded Protein Response Pathway

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Parkinson's disease (PD), the second most prevalent neurodegenerative disorder, is characterized by the degeneration of dopamine (DA) neurons and age-dependent formation of protein inclusions that contain the α -synuclein (α -syn) protein. RNA interference (RNAi) screening using *Caenorhabditis elegans* identified RTCB-1, an uncharacterized gene product, as one of several significant modifiers of α -syn protein misfolding. RTCB-1 is the worm ortholog of the human HSPC117 protein, a component of RNA trafficking granules in mammalian neurons. Here we show that RTCB-1 protects *C. elegans* DA neurons from age-dependent degeneration induced by human α -syn. Moreover, neuronal-specific RNAi depletion of *rtcb-1* enhanced α -syn-induced degeneration. Similar results were obtained when worms were exposed to the DA neurotoxin 6-hydroxydopamine. HSPC117 has been characterized recently as an essential subunit of the human tRNA splicing ligase complex. tRNA ligases have alternative functions in RNA repair and nonconventional mRNA splicing events. For example, in yeast, unconventional splicing of *HAC1*, a transcription factor that controls the unfolded protein response (UPR), is mediated by a tRNA ligase. In *C. elegans*, we demonstrate that RTCB-1 is necessary for *xbp-1* (worm homolog of *HAC1*) mRNA splicing. Moreover, using a RNA ligase-dead mutant, we determine that the ligase activity of worm RTCB-1 is required for its neuroprotective role, which, in turn, is mediated through XBP-1 in the UPR pathway. Collectively, these studies highlight the mechanistic intersection of RNA processing and proteostasis in mediating neuroprotection.

Key words: alpha-synuclein; *C. elegans*; dopamine; neuroprotection; Parkinson's; RNA

Introduction

The pathogenesis of Parkinson's disease (PD) involves the loss of dopamine (DA) neurons in the midbrain and manifests through mechanisms such as mitochondrial defects and protein misfolding, leading to endoplasmic reticulum (ER) stress and proteasome dysfunction (Yacoubian and Standaert, 2009). Another characteristic of PD is the accumulation of α -synuclein (α -syn) into inclusions termed Lewy bodies (Dickson et al., 2009). Mutation or multiplication of the wild-type (WT) α -syn locus causes misfolding of α -syn protein, leading to aggregation (Uversky, 2007). Discerning molecular mechanisms underlying neurodegeneration is a prerequisite for identifying functional modifiers with translational potential for PD.

We have established previously *Caenorhabditis elegans* as an animal model to investigate PD, wherein overexpression of WT

human α -syn driven under a DA neuron-specific promoter causes age- and dose-dependent neurodegeneration (Cao et al., 2005; Hamamichi et al., 2008). This correlates with human genetics, in which multiplication of the WT α -syn locus leads to familial PD (Singleton et al., 2003). Hamamichi et al. (2008) conducted a large-scale RNA interference (RNAi) screen in *C. elegans* that identified several genetic modifiers of α -syn toxicity, including one uncharacterized open-reading frame, F16A11.2, now annotated as *rtcb-1*.

RTCB-1, encoding a 505 aa protein, contains a highly conserved RtcB domain, common to the RtcB family of proteins present in eukaryotes, bacteria, and archaeobacteria. RtcB proteins are atypical RNA ligases with broad substrate specificity implicated in tRNA splicing and repair (Englert et al., 2011; Tanaka et al., 2011; Chakravarty et al., 2012). Human HSPC117 can bind to AU-rich elements within mRNA (Rousseau et al., 2002) and was characterized recently as an essential subunit of the tRNA splicing ligase complex (Popow et al., 2011). Although HSPC117 is ubiquitously expressed in human tissues, a functional role in neurons was suggested by its presence in RNA transport granules in mouse brain extracts (Kanai et al., 2004).

Besides tRNA splicing, RNA ligases have been demonstrated to function in RNA repair and nonconventional mRNA splicing events. One such event is the unconventional splicing of *HAC1* mRNA, encoding a yeast transcription factor that controls the unfolded protein response (UPR). Interestingly, the bacterial homolog of RTCB-1, RtcB, can functionally complement yeast

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tRNA ligase splicing activity (Sidrauski et al., 1996; Tanaka et al., 2011). The UPR pathway is conserved from yeast to mammals (Iwawaki et al., 2001), in which XBP1, a homolog of HAC1, is cleaved by IRE1 endonuclease (Shen et al., 2001). During ER stress, the unspliced *XBP1* transcript is alternatively spliced to produce an active version of *XBP1* mRNA (Calfon et al., 2002). XBP1 mediates transcription of chaperone proteins such as BiP/Grp78 and facilitates the exit and degradation of misfolded proteins via ER-associated protein degradation (ERAD). In *C. elegans*, XBP-1 activates UPR genes involved in folding, secretion, ER biogenesis, and autophagy. There are no homologs of yeast tRNA ligase in either mammals or *C. elegans*, thus the factor responsible for splicing of *XBP1* in metazoan systems has remained unknown (Wang and Shuman, 2005). Here we report RTCB-1 as a functional mediator of *xbp-1* splicing and describe its role in neuroprotection.

Materials and Methods

Plasmid construction. *rtcb-1* and *xbp-1* were amplified from genomic DNA isolated from N2 Bristol nematodes using Phusion high-fidelity polymerase. An N-terminal FLAG tag sequence was added to *rtcb-1*/*xbp-1* cDNA during the PCR amplification process. Using Gateway Technology (Life Technologies), PCR-amplified constructs were cloned into plasmid entry vector pDONR221 by BP reaction, and the constructs were further cloned into Gateway expression vector pDEST-DAT-1 (Cao et al., 2005). To generate the point mutant RTCB-1 C122A, TagMaster site-directed mutagenesis kit (GM Biosciences) was used. The constructs were verified by DNA sequencing.

***C. elegans* strains.** Nematodes were maintained using standard procedures (Brenner, 1974). We obtained the following strains from the Caenorhabditis Genetics Center: *rtcb-1(gk451)* and *xbp-1(zc12)*. Strain BY250 [*vtIs7*(*P_{dat-1}::GFP*)] was a generous gift from Randy Blakely (Vanderbilt University, Nashville, TN). The isogenic strain UA44 [*baIn11*(*P_{dat-1}:: α -syn*, *P_{dat-1}::GFP*)] expresses α -syn and GFP in the DA neurons. UA196 [*sid-1(pk3321)*; *baIn33*(*P_{dat-1}::*sid-1**, *P_{myo-2}::mCherry*); *baIn11*], UA197 [*sid-1(pk3321)*; *uIS69*(*P_{unc-119}::*sid-1**, *P_{myo-2}::mCherry*); *baIn11*], and UA202 [*sid-1(pk3321)*; *baIn36*(*P_{dat-1}::*sid-1**, *P_{myo-2}::mCherry*); *vtIs7*(*P_{dat-1}::GFP*)] were generated as described previously (Harrington et al., 2012). UA196 expresses α -syn, GFP, and SID-1 in the DA neurons. UA202 expresses only GFP and SID-1 in the DA neurons. Both are sensitive to RNAi specifically in the DA neurons. UA197 is a pan-neuronal specific RNAi strain expressing α -syn and GFP in DA neurons and SID-1 in all neurons. Three independent stable transgenic lines were generated by injecting *P_{dat-1}::rtcb-1*, along with a phenotypic marker (*P_{unc-54}::mCherry*), into UA44 hermaphrodites. These lines express RTCB-1 in the DA neurons and mCherry in the body wall muscle cells, as a phenotypic marker. One of the stable lines, with the most significant level of neuroprotection against α -syn-induced degeneration (data not shown), was further integrated into the *C. elegans* genome by UV irradiation (Inoue and Thomas, 2000) to create strain UA266 [*baIn48*(*P_{dat-1}::rtcb-1*; *P_{unc-54}::mCherry*); *baIn11*]. The isolated homozygous integrated line was used for neuroprotection analysis in Figure 1A. UA266 was further crossed into strain UA197 to generate UA267 [*baIn48*; *baIn11*; *sid-1(pk3321)*; *uIS69*], which was used for neuroprotection analysis in Figure 4A. *sid-1(pk3321)* homozygosity was maintained as described previously (Harrington et al., 2012). This strain expresses α -syn, GFP, and RTCB-1 in the dopaminergic neurons and is highly selective for RNAi in the neurons. To create UA270 [*baEx159*(*P_{dat-1}::*xbp-1u**; *rol-6(su1006)*); *sid-1(pk3321)*; *uIS69*; *baIn11*], analyzed in Figure 4B, *P_{dat-1}::xbp-1u* and pRF4 *rol-6(su1006)* were injected into UA197. Strain UA269, analyzed in Figure 5, consists of [*baEx158*(*P_{dat-1}::rtcb-1(C122A)*, *rol-6(su1006)*); *baIn11*].

RNAi treatments. Bacterial RNAi feeding constructs of *rtcb-1* and *xbp-1* were obtained from the Ahringer *C. elegans* library (Kamath et al., 2003); they were isolated and grown overnight in Luria broth media containing 100 μ g/ml ampicillin. Nematode growth medium (NGM) plates containing 0.25% β -D-lactose were seeded with 250 μ l of RNAi culture and allowed to dry overnight. Ten dauer or L1-stage worms [N2,

or neuron-specific RNAi worm strains (UA196, UA197, UA266 or UA267, UA270)] were transferred to the plates and grown at 20°C until adulthood. Adult worms were then transferred to corresponding freshly made RNAi plates and allowed to lay eggs for 6 h to synchronize. For neurodegeneration assays, the DA neurons in the F1 progeny of the RNAi-treated worms were scored at days 4, 6, 7 or day 10 after hatching, as described below. For quantitative PCR (qPCR) studies, RNAi-treated late L4 (larval stage) N2 and/or UA202 worms were treated with 10 μ g/ml tunicamycin for 6 h and then collected for RNA isolation, as described below.

***C. elegans* neurodegeneration assay.** Worms were analyzed for DA neurodegeneration as described previously (Cao et al., 2005). Briefly, worms (strains UA196, UA197, UA266, or UA267) were synchronized, grown at 20°C, and scored for α -syn-induced DA neurodegeneration at days 4, 6, 7 or day 10 after hatching. Worms were considered normal when all six anterior DA neurons [four CEP (cephalic) and two ADE (anterior deirid)] were present without any visible signs of degeneration. If a worm displayed degeneration in at least one of the six neurons, it was scored as exhibiting degeneration. In total, at least 90 adult worms were analyzed for each independent transgenic line or RNAi treatment. 6-Hydroxydopamine (6-OHDA) assay was performed as described previously (Nass et al., 2002). Briefly, L4 worms (UA202) were washed with ddH₂O three times and treated with 30 mM 6-OHDA (Tocris Bioscience) containing 1 mM ascorbic acid, followed by gentle agitation for 1 h. Subsequently, the worms were again washed and put onto freshly made RNAi plates until analysis.

RNA isolation from *C. elegans*. For each independent sample, total RNA was isolated from 50 young adult hermaphrodite worms as described previously (Hamamichi et al., 2008). Briefly, the worms were washed three times with M9 buffer (3 g of KH₂PO₄, 12.8 g of Na₂HPO₄, 5 g of NaCl, 1 ml of 1 M MgSO₄, and H₂O to 1 L) and then frozen at –80°C until use. After thawing, 500 μ l of TRI Reagent (Molecular Research Center) was added to the samples, followed by brief vortex and incubated at room temperature (RT) for 10 min. The samples were then subjected to repeated freeze–thaw cycling for four times in liquid N₂. The phases were separated by adding 50 μ l of 1-bromo-3-chloropropane (Acros Organics) with 15 s of vortexing. The samples were then incubated at RT for 15 min and centrifuged at 13,000 \times g at 4°C for 15 min. The supernatant was then transferred to an RNase-free microcentrifuge tube, mixed with 1.5 μ l of glycoblue (Ambion) and 250 μ l of –20°C chilled isopropanol, and then stored overnight at –20°C. After incubation, the sample was centrifuged at 13,000 \times g at 4°C for 15 min, and the supernatant was discarded. The pellet was washed with 500 μ l of RNase-free ethanol (75%), air dried for 5 min, and resuspended in 10 μ l of nuclease-free water. Samples were then treated with 1 μ l of DNase I and 1 μ l of DNase I buffer (Promega) at 37°C for 15 min and then for 10 min with 1 μ l of DNase stop at 65°C. Total RNA was quantitated using a Nanodrop and cDNA was synthesized using iScript cDNA synthesis kit (Bio-Rad) following the protocols of the manufacturer.

Real-time qPCR. qPCR reactions were performed using IQ SYBR Green Supermix (Bio-Rad) with the CFX96 Real-Time System (Bio-Rad) as described previously (Thompson et al., 2014). The following previously described primer sequences were used: spliced *xbp-1*, total *xbp-1*, and *hsp-4* (Richardson et al., 2010); and *tba-1*, *ama-1*, and *gpd-2* (Hoogewijs et al., 2008). For the remaining primers, full-length gene sequences were obtained from WormBase, and primers were designed by the Primer3 software and evaluated for potential secondary structures of the amplicon by MFOLD software. MFOLD analysis was performed by adjusting the values to 50 mM Na⁺, 3 mM Mg²⁺, and 60°C annealing temperature.

The following *C. elegans* primers were used: spliced *xbp-1* forward, TGCCCTTGAATCAGCAGTGG; spliced *xbp-1* reverse, ACCGCTCTGCTCCTTCCTCAATG; unspliced *xbp-1* forward, AGAAGTCGTCGTGAGGTTG; unspliced *xbp-1* reverse, CCTGTCCCACTGCTGAG; total *xbp-1* forward, CCGATCCACCTCCATCAAC; total *xbp-1* reverse, ACCGCTCTGCTCCTTCCTCAATG; *hsp-4* forward, AGTTGAAATCATCGCCAACG; *hsp-4* reverse, GCCCAATCAGACGCTTGG; *rtcb-1* forward, GGAAGTCGAGGACTTGGACA; *rtcb-1* reverse, GTTAACCCAGGCGAAGTTTG; *tba-1* forward, GTACACTCCACTGATCTCTGCTGACAAG; *tba-1* reverse, CTCTGTACAAGAGGCAAACAGCCATG; *ama-1* forward, CCTACGATGTATCGAGGCAAA; *ama-1* reverse, CCTCCCTCCGGTGAATAATG; *gpd-2* forward, CTCCATCGACTA-

CATGGTCTACTTG; *gpd-2* reverse, AGCTGG GTCTCTTGAGTTGTAGAC.

PCR efficiency was calculated from standard curves that were generated using serial dilutions of cDNA of all samples. All targeted genes were measured in triplicate, and three independent biological replicates were tested for each sample. No amplification was detected in no template and no reverse transcriptase controls. The Cq quantification cycle values recorded by CFX Manager Software version 3.0 (Bio-Rad) were exported into qBase^{PLUS} version 2.6 (Biogazelle) for determining reference target stability (GeNorm $M < 0.5$, $CV < 0.2$). *ama-1*, *tba-1*, and *gpd-2* were used as internal controls. Relative mRNA expression levels were normalized using these reference control genes.

Tunicamycin treatment of *hsp-4::GFP* worms. RNAi-treated [empty vector (EV), *rtcb-1* or *xbp-1*] late L4-stage animals were transferred to NGM plates spread with gradient concentrations of tunicamycin: 0, 2, 4, 8, 12, and 16 $\mu\text{g}/\text{ml}$ for an exposure time of 6 h, followed by *hsp-4::GFP* analysis, as described in the next section. Tunicamycin (Calbiochem) was made at a stock concentration of 1 mg/ml in DMSO.

Imaging and statistics. Fluorescent microscopy was performed using a Nikon Eclipse E800 epifluorescence microscope equipped with an Endow GFP or Texas Red HYQ filter cube (Chroma Technology). A Cool Snap CCD camera (Photometrics) driven by MetaMorph software (Molecular Devices) was used to acquire images. Using established methods for *hsp-4::GFP* transcriptional fusion reporter analysis (Chen et al., 2010), each animal was imaged in a consistent anatomical region (at the intestinal region directly behind the pharynx) at the same magnification and exposure intensity. The pixel intensity was quantified within a $100 \times 100 \mu\text{m}$ box drawn in the same region and compiled across three replicates. For statistical analyses of all the datasets, either a Student's *t* test or one-way ANOVA followed by a Tukey's *post hoc* was used.

Results

RTCB-1 rescues age-dependent DA neuron loss induced by α -syn overexpression in *C. elegans*

Previously, a large RNAi screen revealed that depletion of *rtcb-1* caused enhanced α -syn misfolding in *C. elegans* body wall muscle cells (Hamamichi et al., 2008). Alignment of amino acid sequences between metazoan RtcB orthologs reveals the strongly conserved nature of these proteins (Popow et al., 2011). A sequence alignment of human HSPC117 with *C. elegans* RTCB-1 shows 73% amino acid sequence identity.

We wanted to determine whether RTCB-1 would protect DA neurons from age-dependent decline from α -syn-induced neurodegeneration. Transgenic animals expressing *C. elegans* *rtcb-1* cDNA under the control of DA neuron-specific promoter ($P_{dat-1}::rtcb-1$) were injected into worms expressing both α -syn and GFP in DA

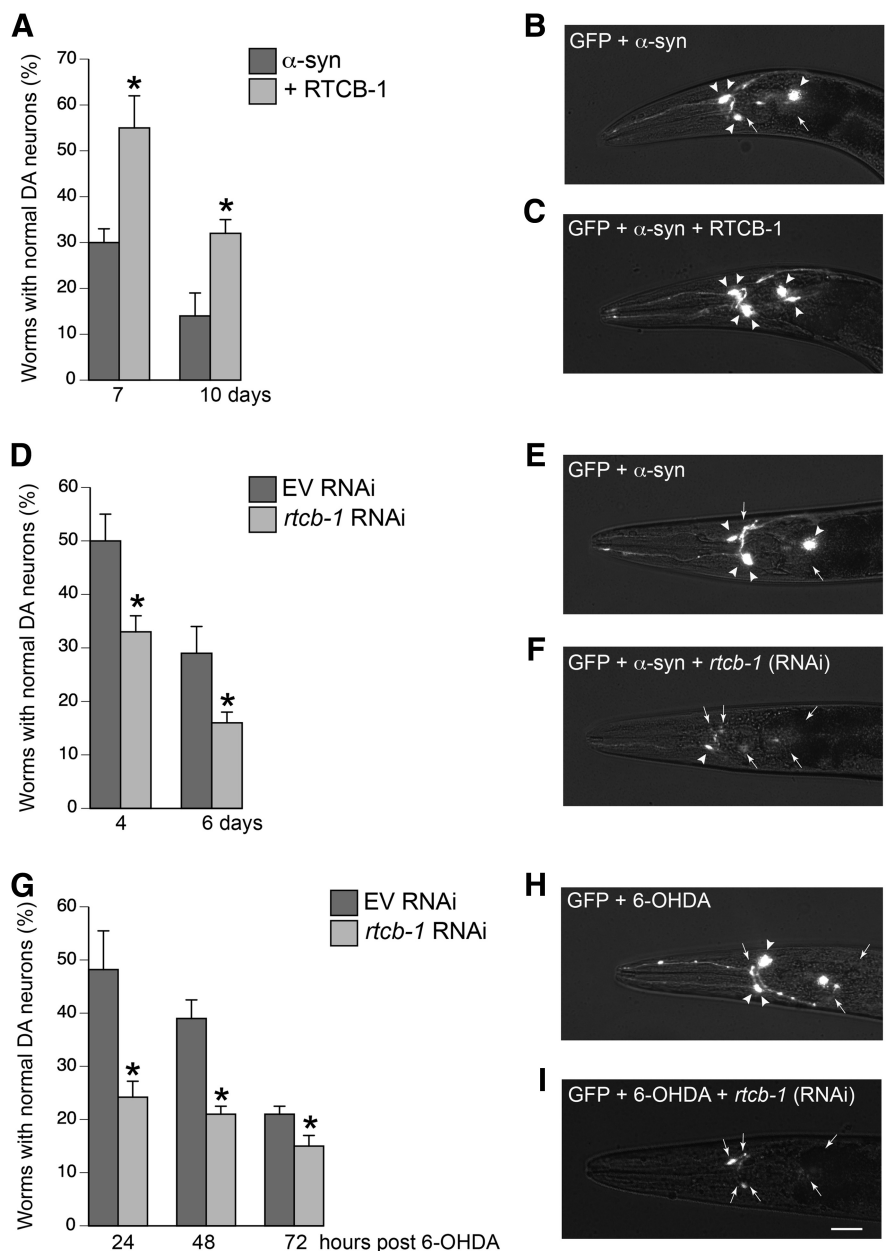


Figure 1. RTCB-1 is neuroprotective against α -syn or 6-OHDA-induced toxicity. **A**, RTCB-1 overexpression protects *C. elegans* DA neurons from α -syn-induced neurodegeneration at 7- and 10-d worm stages compared with the α -syn control. **B**, **C**, Representative images of the neuroprotection assay described in **A**. **B**, Worm expressing GFP and α -syn specifically in the six anterior DA neurons with four neurons intact (arrowheads) and two neurons degenerated (arrows). **C**, Worm coexpressing RTCB-1 shows neuroprotection with all six neurons protected (arrowheads). **D**, RNAi depletion of *rtcb-1* enhances DA neurodegeneration caused by α -syn at 4 and 6 d worm stages when compared with EV α -syn control. **E**, **F**, Representative worm images of the RNAi experiment described in **D**. **E**, Representative RNAi-treated (EV) worm expressing α -syn missing two neurons (arrows). **F**, RNAi knockdown of *rtcb-1* shows increased degeneration with loss of all five of six anterior DA neurons (arrows). **G**, RNAi depletion of *rtcb-1* accelerates DA neurodegeneration caused by 6-OHDA at 24, 48, and 72 h after 1 h 6-OHDA treatment when compared with 6-OHDA-treated EV control. **H**, Exemplar worm exposed to 6-OHDA, in this case missing three DA neurons (arrows). **I**, A worm treated with *rtcb-1* RNAi and 6-OHDA is missing all six neurons, indicative of enhanced degeneration (arrows). Data are reported as the mean \pm SD, $n = 30$ worms per trial for a total of three replicates. * $p < 0.05$, Student's *t* test. Scale bar, 20 μm .

neurons. Overexpression of α -syn alone resulted in significant degeneration of DA neurons whereby only 30 and 14% of the population displayed normal neurons at day 7 and day 10 of the worm lifespan, respectively, indicating an age-dependent degeneration, as reported previously (Hamamichi et al., 2008). Animals coexpressing RTCB-1 and α -syn exhibited significant rescue of DA neurodegeneration at both time points (Fig. 1A). Representative im-

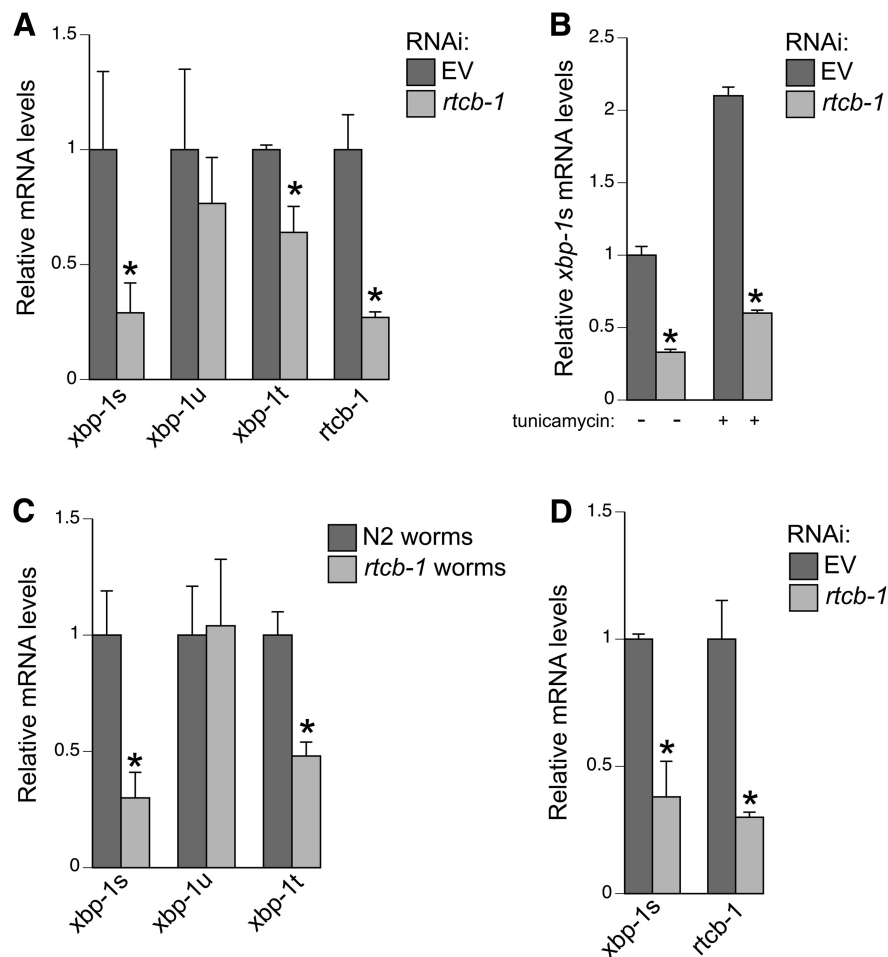


Figure 2. RTCB-1 regulates *xbp-1* splicing in *C. elegans*. qPCR-based detection of *xbp-1s*, *xbp-1u*, *xbp-1t*, or *rtc-1* mRNA expression in RNAi-treated and mutant worm strains. **A**, Tunicamycin (10 μ g/ml)-treated N2 worms depleted for *rtc-1* RNAi display suppressed spliced and total levels of *xbp-1* when compared with EV control. The unspliced levels of *xbp-1* are similar in both RNAi treatments. Primers specific to *rtc-1* were used to confirm knockdown. **B**, A comparison of spliced *xbp-1* mRNA levels in RNAi-treated N2 worms in the absence and presence of tunicamycin exposure. **C**, Likewise, heterozygous *rtc-1* mutant worms (strain VC1094) treated with tunicamycin showed a significant decrease in *xbp-1s* and *xbp-1t* when compared with N2 WT worms. **D**, DA neuron-specific RNAi knockdown of *rtc-1* also significantly decreased *xbp-1s* levels in worms (strain UA202) treated with tunicamycin. We confirmed specific knockdown using primers specific to *rtc-1*. Relative mRNA expression levels were normalized to the control. Error bars represent \pm SD from three different experiments. * $p < 0.05$, Student's *t* test.

ages depict the neuroprotective effect conferred by RTCB-1 *in vivo* (Fig. 1B vs C).

DA neuronal-specific depletion of *rtc-1* enhanced α -syn- and 6-OHDA-induced degeneration

As might be expected for a ubiquitous gene product associated with critical cellular processes, a putative *C. elegans* *rtc-1* homozygous null mutant causes sterility. Therefore, to further investigate the neuroprotective properties of RTCB-1, we used a method of neuron-selective RNAi knockdown specifically in DA neurons of *C. elegans* to assay for neurodegeneration using a DA neuronal-specific RNAi strain expressing α -syn and GFP in the DA neurons (Harrington et al., 2012). Loss of RTCB-1 further enhanced DA degeneration caused by α -syn at day 4 and day 6 post hatching, when compared to RNAi EV control (Fig. 1D). Figure 1, E and F, shows representative images of an RNAi-treated worm expressing α -syn and GFP in the DA neurons (EV control) and an *rtc-1* RNAi-treated worm expressing α -syn and GFP, exhibiting enhanced DA degeneration.

To determine whether the neuroprotective effect of RTCB-1 was specific to α -syn, we examined vulnerability to a neurotoxin, 6-OHDA, in worms. Selective degeneration of DA neurons occurs in *C. elegans* after exposure to 6-OHDA (Nass et al., 2002). Using another DA neuronal-specific RNAi-sensitive strain, expressing only GFP in DA neurons, worms were knocked down with dsRNA targeting *rtc-1* or EV and subjected to 6-OHDA treatment. Animals treated with 6-OHDA and EV RNAi exhibited degeneration of DA neurons at 24, 48, and 72 h after exposure (Fig. 1G). Cotreatment with *rtc-1* RNAi caused additional enhancement in DA degeneration at all time points evaluated (Fig. 1G). Representative images of *C. elegans* DA neurons as a result of 6-OHDA exposure with EV or *rtc-1* RNAi treatment are shown in Figure 1, H and I.

RTCB-1 is required for *xbp-1* mRNA splicing

According to a recent study in *Saccharomyces cerevisiae*, the bacterial homolog RtcB acts as an RNA repair enzyme and has the capacity to replace the function of Trl1 (yeast RNA ligase) as a catalyst of unconventional HAC1 mRNA splicing during the UPR (Tanaka et al., 2011). Similar unconventional splicing has also been described for the gene *XBPI* in humans, mice, *C. elegans*, and *Drosophila melanogaster* (Hooks and Griffiths-Jones et al., 2011). The unconventional splicing of *XBPI* is catalyzed by an endonuclease, IRE1, and an undescribed RNA ligase. Based on these studies, we hypothesized that RTCB-1 could represent the RNA ligase required for *xbp-1* splicing in *C. elegans*. Spliced *xbp-1* level increases in response to tunicamycin in WT (N2)

worms, and the level of *xbp-1* is reduced in *xbp-1* RNAi worms under both basal and tunicamycin-treated conditions (Chen et al., 2010). To detect a role for RTCB-1 in *xbp-1* splicing, we performed RNAi knockdown of *rtc-1* in N2 worms and measured spliced (*xbp-1s*), unspliced (*xbp-1u*), and total (*xbp-1t*; i.e., *xbp-1s* + *xbp-1u*) levels of *xbp-1* mRNA, using real-time qPCR. Under tunicamycin-treated conditions, knockdown of *rtc-1* significantly lowered *xbp-1s* and *xbp-1t* mRNA expression compared with control (Fig. 2A). Notably, *xbp-1u* levels were unaffected in RNAi versus control samples. *rtc-1* primers were used to ensure its knockdown in these treated worms (Fig. 2A). Under nonstressed (no tunicamycin) conditions, knockdown of *rtc-1* also significantly lowers the basal level of *xbp-1s* compared with EV control (Fig. 2B).

We further confirmed these results using an *rtc-1* mutant strain, which as a homozygote is sterile and has limited viability; thus, we maintained and analyzed it as a heterozygote. The *rtc-1* mutant *gk451* consists of a 370 bp deletion spanning the promoter and the first exon, most likely resulting in a nonfunctional

protein. *rtcb-1* mutant animals were grown until day 4 after hatching and exposed to tunicamycin (for 6 h) along with N2 control worms. Based on qPCR results, *xbp-1s* and *xbp-1t* mRNA expression was significantly reduced in *rtcb-1* mutant worms compared with control animals (Fig. 2C). It should be noted that *xbp-1* mutant animals, containing a nonsense mutation in the XBP-1 predicted protein, are unable to mount a UPR (Bischof et al., 2008; Chen et al., 2010). These combined RNAi and mutant analyses support the hypothesis that RTCB-1 regulates *xbp-1* splicing.

We next wanted to determine whether RTCB-1-mediated splicing of *xbp-1* mRNA was extended to DA neurons. To examine a neuron-specific effect of *xbp-1* splicing in *C. elegans*, we used a selectively DA neuronal-sensitive strain to knock-down *rtcb-1* and measured *xbp-1* mRNA levels. Using *rtcb-1*-specific primers, we first determined whether the knockdown of *rtcb-1* in the eight DA neurons of *C. elegans* hermaphrodites could be assessed successfully via qPCR. The results indicated sufficient sensitivity to detect a significant decrease in the expression levels of *rtcb-1* mRNA in RNAi-treated worms when compared with EV control (Fig. 2D). Furthermore, expression of *xbp-1s* was substantially lowered in *rtcb-1* RNAi worms compared with the control. Therefore, it is clear that RTCB-1 also functions *in vivo* to mediate *xbp-1* splicing in DA neurons.

RNAi knockdown of *rtcb-1* lowers ER stress response *in vivo*

In PD, as well as other neurodegenerative diseases, the presence of misfolded proteins disrupts the balance between the synthesis of new proteins and the ability of the ER to process these proteins. As a result, ER stress in turn provokes the UPR, which may protect cells against the toxic accumulation of misfolded proteins (Harding et al., 2002). To examine the function of RTCB-1 during ER stress-induced UPR, an *in vivo* quantitative readout assay for the ER stress response was performed. Levels of ER stress was monitored in transgenic animals using a fluorescent reporter, GFP, under the control of *hsp-4* promoter ($P_{hsp-4}::GFP$). HSP-4 (worm homolog of BiP) is one of the ER chaperones regulated by an active *xbp-1* (Calfon et al., 2002). During tunicamycin-initiated ER stress, transcription of *hsp-4* is induced and GFP is highly expressed, thereby providing a direct and quantitative measure of the *in vivo* stress response. A previous study has shown that *hsp-4::GFP* worms treated with *xbp-1* RNAi (in the presence of tunicamycin) exhibit reduced GFP intensity compared with controls (Chen et al., 2010). To determine the role of RTCB-1 in ER stress response, worms fed with *rtcb-1* RNAi (or *xbp-1* RNAi as a positive control) were treated using a gradient of tunicamycin concentrations and examined by fluorescent intensity changes. As expected, a significant

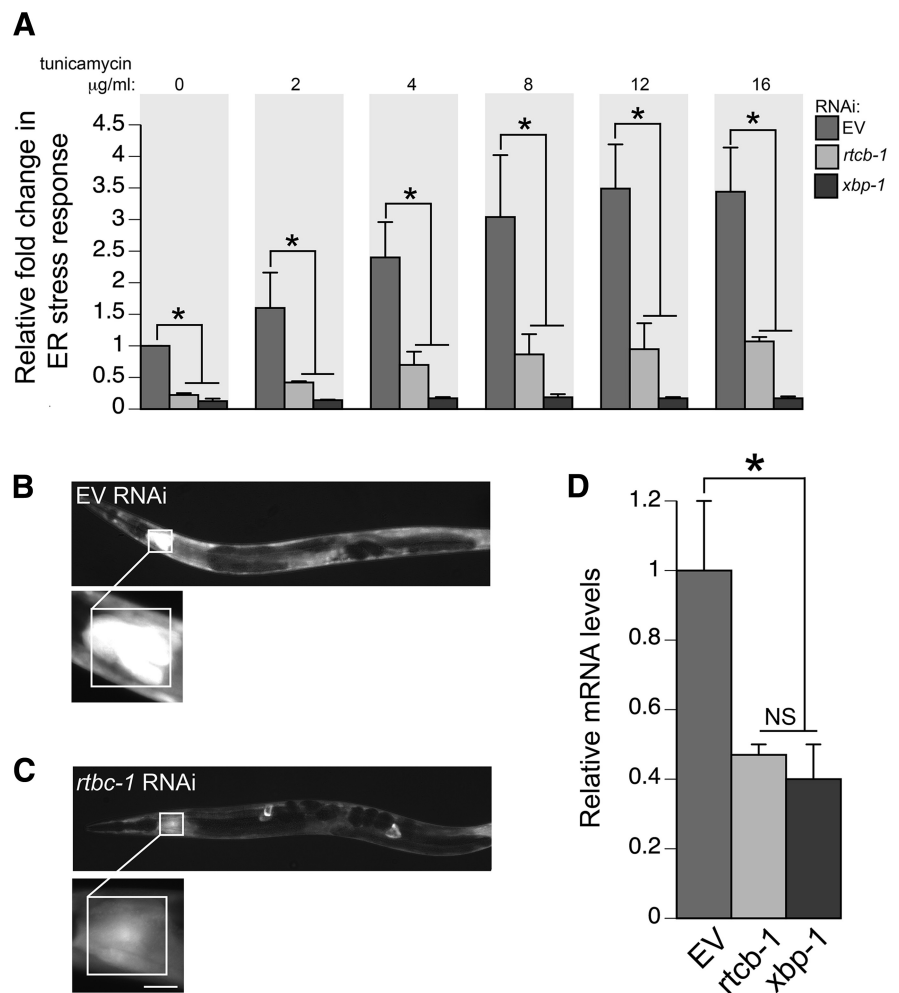


Figure 3. Knockdown of RTCB-1 lowers ER stress response. **A**, Bar charts of GFP intensity from the stress reporter *hsp-4::GFP* alone (with EV RNAi) or after RNAi knockdown of *rtcb-1* or *xbp-1* measured consistently in the regions shown in **B** and **C**. Strains were treated with increasing concentrations of tunicamycin for 6 h (or DMSO as control). Data presented have been normalized to EV RNAi, untreated, *hsp-4::GFP* samples and is calculated as the mean \pm SD of three experiments, in which 30 animals were analyzed per replicate ($p < 0.05$, one-way ANOVA). RNAi knockdown of both *rtcb-1* and *xbp-1* (positive control) caused significant downregulation of *hsp-4::GFP* expression. **B**, **C**, Representative *C. elegans* images for RNAi treatments described in **A**. Pixel intensities were measured in a $100 \times 100 \mu\text{m}$ region of the intestine proximal to the pharynx. The region highlighted in the white box shows GFP expression driven by the *hsp-4* gene promoter that was measured in all animals. Scale bar, $50 \mu\text{m}$. **D**, RNAi depletion of *rtcb-1* and *xbp-1* (positive control) in N2 worms caused a decrease in *hsp-4* mRNA expression when compared with EV control, as shown by qPCR ($*p < 0.05$, one-way ANOVA). Values are the mean \pm SD of three independent experiments and were normalized to the control.

decrease in *hsp-4::GFP* expression was observed for both *xbp-1* RNAi and *rtcb-1* RNAi compared with the EV RNAi control at all concentrations tested (Fig. 3A–C). We also performed qPCR to measure the mRNA expression of *hsp-4* in these RNAi-treated worms that were exposed to $10 \mu\text{g/ml}$ tunicamycin. Similar to the *in vivo* results, the levels of *hsp-4* expression in *rtcb-1* and *xbp-1* RNAi worms were lower than that of the EV RNAi treated animals (Fig. 3D). These data further strengthen the genetic link between RTCB-1 activity and *xbp-1* in regulating ER stress response.

RNAi depletion of *xbp-1* blocks the neuroprotective function of RTCB-1

Having revealed a role for RTCB-1 in *xbp-1* mRNA splicing, we sought to determine whether its neuroprotective function is mediated by XBP-1. *In vitro* studies in SH-SY5Y cells and *in vivo* studies in mice have shown that overexpression of *xbp-1s* protects

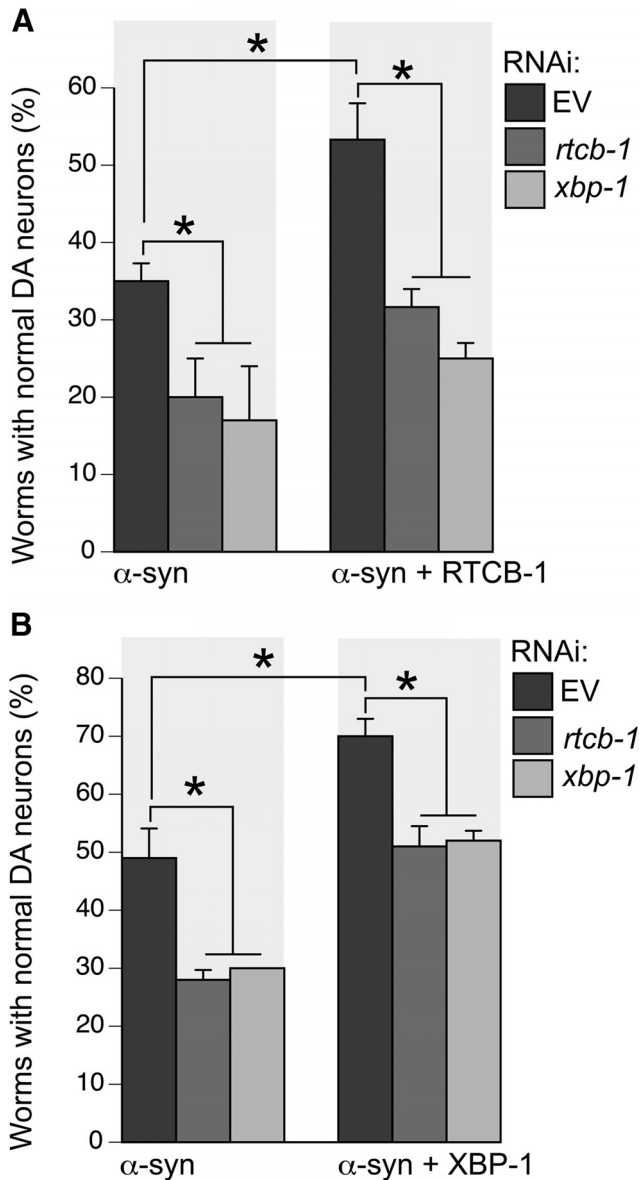


Figure 4. RTCB-1-induced neuroprotection against α -syn is mediated by XBP-1 signaling. **A**, Pan-neuronal-specific knockdown of *rtcb-1* or *xbp-1* enhances α -syn-induced neurodegeneration when compared with the EV control. These worms expressing α -syn and GFP were then crossed with worms expressing RTCB-1. Overexpression of RTCB-1 protects DA neurons from α -syn-induced toxicity in worms treated with RNAi EV. RNAi knockdown of *rtcb-1* or *xbp-1* failed to protect neurons from α -syn-induced degeneration in an RTCB-1 overexpression background. **B**, Overexpression of XBP-1 protects DA neurons from α -syn-induced toxicity in worms treated with RNAi EV when compared with α -syn control. Pan-neuronal-specific knockdown of *rtcb-1* blocks the neuroprotective phenotype of XBP-1. DA neuron analyses were performed at day 6 after hatching. Data are reported as the mean \pm SD, $n = 30$ worms per trial for a total of three replicates. * $p < 0.05$, one-way ANOVA.

DA neurons from dying from MPTP (Sado et al., 2009). Here we used a pan-neuronal-sensitive RNAi strain expressing α -syn and GFP in DA neurons to perform RNAi knockdown of *xbp-1* and *rtcb-1* separately. Strikingly, we observed a significant change in neurodegeneration in each of them compared with the α -syn control (Fig. 4A). To examine the genetic interaction between RTCB-1 and *xbp-1* in regulating neuroprotection, we crossed our pan-neuronal-specific RNAi strain (expressing α -syn and GFP in DA neurons) with worms overexpressing RTCB-1 in DA neurons ($P_{dat-1}::rtcb-1$) to generate a pan-neuronal-selective RNAi-

sensitive strain expressing α -syn, RTCB-1, and GFP in DA neurons. Analysis of this new strain showed that RTCB-1 overexpression attenuated the DA neuron loss induced by α -syn, consistent with our previous results using a non-RNAi-specific strain (Fig. 4A). By using this strain for RNAi analysis, we further discerned that *xbp-1* knockdown suppressed the neuroprotective phenotype attained through RTCB-1 overexpression (Fig. 4A). Thus, RTCB-1-mediated neuroprotection requires XBP-1.

To further support this conclusion, we overexpressed XBP-1 in DA neurons of worms expressing α -syn and observed significant neuroprotection with the endogenous level of *rtcb-1* (Fig. 4B). Alternately, knocking down *rtcb-1* in this XBP-1 overexpression background caused a reversal of the neuroprotective phenotype (Fig. 4B); this was anticipated, because loss of *rtcb-1* would block the splicing of *xbp-1* mRNA (as already shown in Fig. 2). Together, these results suggest that RTCB-1-induced neuroprotection is mediated through XBP-1 signaling. Whereas loss of *xbp-1* negates the neuroprotective phenotype of RTCB-1, loss of *rtcb-1* blocks the neuroprotective phenotype of XBP-1 on α -syn-induced degeneration.

Mutation of a conserved residue abolishes neuroprotective activity of RTCB-1

Previously, it has been shown that mutation of a conserved residue (C122A) in the human RTCB-1 homolog HSPC117 abolished its RNA ligase activity (Popow et al., 2011). To investigate whether putative RNA ligase activity of RTCB-1 is required for its neuroprotection, we created a ligase-dead version of *C. elegans* RTCB-1 by mutating the same site in the worm homolog, which contains a conserved residue at the same amino acid position (Cys¹²² to Ala¹²²). The *rtcb-1* (C122A) variant was overexpressed in DA neurons of worms also expressing α -syn. Unlike WT *rtcb-1*, this mutated version was unable to provide significant neuroprotection when compared with α -syn alone as worms aged (compare Figs. 5 and 1).

Discussion

Humans, bacteria, and archaea all have RTCB-1 homologs, thus showing the evolutionary conservation of this protein. These proteins are characterized to be novel RNA ligases implicated in tRNA splicing and repair (Englert et al., 2011; Popow et al., 2011; Tanaka et al., 2011; Chakravarty et al., 2012). However, with the exception of the *C. elegans* ortholog, these proteins have not been correlated directly with neuronal function or survival to date. In the study by Kanai et al. (2004), the human homolog HSPC117 was identified to be a component of RNA transporting granule, thereby suggesting an overall role in RNA processing or RNA metabolism. The high degree of conservation among RtcB proteins suggests a shared function for *C. elegans* RTCB-1 with other family members. In this context, we determined that RTCB-1, a previously uncharacterized protein in *C. elegans*, is a neuroprotective gene product that regulates *xbp-1* mRNA splicing *in vivo*. Likewise, it is evident that the underlying mechanism associated with RTCB-1-mediated neuroprotection is based on XBP-1-mediated UPR signaling.

Conventional splicing is catalyzed by the spliceosome and takes place exclusively in the nucleus (Tarn and Steitz, 1997). In contrast, the unconventional splicing of *HAC1* and *XBP1* is mechanistically distinct and is completely independent of the spliceosome in that it is catalyzed by IRE1 endonuclease and an RNA ligase (Sidrauski et al., 1996; Kawahara et al., 1998; Gonzalez et al., 1999; Yoshida et al., 2001). Furthermore, unconventional splicing of yeast *HAC1* and *XBP1* mRNAs occurs in the

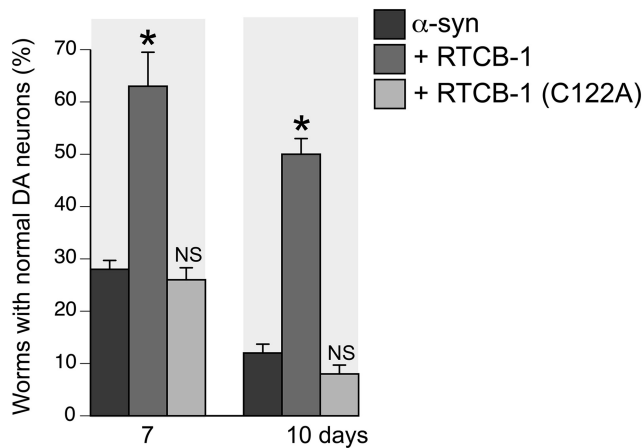


Figure 5. Overexpression of *rtcb-1* (C122A) in DA neurons is not protective from α -syn-induced neurodegeneration. Analysis of worm RTCB-1 mutant at days 7 and 10 failed to show any significant protection of DA neurons when compared with α -syn control, yet overexpression of WT RTCB-1 was protective.

cytoplasm during the UPR (Ruegsegger et al., 2001; Uemura et al., 2009). Notably, there are no RtcB homologs in yeast or plants, but bacterial RtcB can complement the function of yeast tRNA ligase in *HAC1* mRNA splicing (Tanaka et al., 2011).

In mammals, along with the conserved IRE1 signaling pathway, two additional UPR pathways have evolved to maintain proteostasis. These two pathways are respectively mediated by pancreatic-enriched ER kinase (PERK), which facilitates translational inhibition to reduce protein production, and activating transcription factor 6 (ATF6), which mediates transcription to increase the levels of chaperone proteins (Haze et al., 1999; Sood et al., 2000). The coordination of these three pathways provides protection against ER stress in mammals. *C. elegans* has homologs of the vertebrate UPR components IRE1, ATF6, and PERK (Shen et al., 2001; Urano et al., 2002). It also retains the essential function of these components in upregulating the expression of UPR target genes during ER stress. Worms treated with ER stress inducers, such as tunicamycin, thapsigargin, or DTT, exhibit an increase of spliced or stressed *xbp-1s* (*xbp-1s*), followed by activation of UPR genes involved in ER biogenesis (Shen et al., 2001; Calfon et al., 2002). The requirement for the UPR pathway in *C. elegans* is cell type and age dependent, and expression of XBP-1 is elevated in neurons during development (Hetz et al., 2008). Studies have shown that *C. elegans* XBP-1 functions in the nervous system and loss of *xbp-1* blocks UPR signaling (Calfon et al., 2002). Therefore, our data showing the neuroprotective function of RTCB-1 and its role in regulation of UPR *xbp-1* splicing further strengthens our conclusions. For additional studies, it will be instructive to determine a putative neuronal role of RTCB-1 in mitochondrial stress response, because both the ER and mitochondria coordinate the UPR.

Misfolded proteins and associated ER stress are common features of neurodegenerative diseases. Overexpression of mutant forms of α -syn in cultured neuronal cells leads to proteasomal dysfunction and DA neurodegeneration (Stefanis et al., 2001; Tanaka et al., 2001). We reported previously that α -syn aggregation impairs vesicle trafficking and degradation of selective ERAD substrates and causes ER stress (Cooper et al., 2006). Neurotoxins, such as 6-OHDA and MPTP, also cause dopaminergic neuronal death and trigger the activation of genes involved in ER stress and the UPR (Ryu et al., 2002; Holtz and O'Malley, 2003). Inactivation of the UPR leads to organelle dysfunction and cell

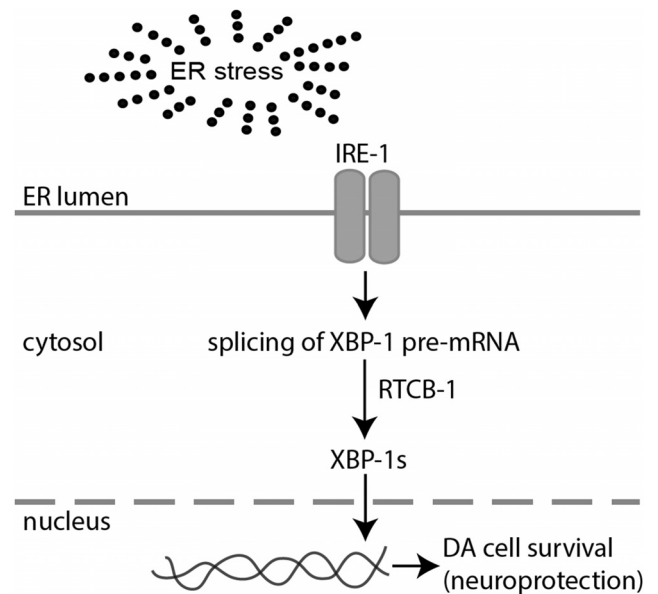


Figure 6. Proposed model for RTCB-1-mediated DA neuroprotection in *C. elegans*. This tentative model describes our current understanding of the underlying mechanism involved in RTCB-1-induced neuroprotection against DA neuron cell death caused by various stressors. We have determined previously that RTCB-1 protects cells from α -syn protein misfolding or α -syn-induced neuronal toxicity. It is possible that, during ER stress caused by accumulation of such toxic misfolded or unwanted proteins, RTCB-1 acts as an RNA modifier that regulates the splicing of the XBP-1 transcription factor in UPR. This in turn activates downstream factors, such as ER chaperones and the hexosamine biosynthetic pathway, and stimulates cytoprotective mechanisms to reestablish protein homeostasis (Denzel et al., 2014). In our *C. elegans* PD model, cells depleted with *rtcb-1* might block the UPR pathway because of a failure in *xbp-1* splicing, leading to cell death or DA neuronal death caused by ER overload. Alternatively, overexpression of RTCB-1 might lead to prolonged *xbp-1* splicing, thereby enhancing proteostasis activity, maintaining ER stress response, and thus protecting against neuronal damage caused by α -syn. Based on our neurodegeneration and splicing assay, the association between RTCB-1 and XBP-1 could be direct or indirect in the regulation of the UPR pathway.

death. For example, deletion of PERK impairs cell survival, whereas increased PERK activity is protective (Harding et al., 2000; Scheuner et al., 2001; Lu et al., 2004). Similarly, IRE1–XBP1 signaling has a protective role during ER stress. *In vitro* ectopic expression of XBP1s activated various secretory pathway genes, increased cell size, and elevated total protein synthesis (Shaffer et al., 2004). XBP-1 also plays a role in controlling the autophagic clearance of aggregated proteins (Hetz et al., 2009). A recent study reported that silencing of XBP1 in mice led to ER stress and DA neurodegeneration, indicating the functional requirement of the UPR in maintaining neuronal proteostasis (Valdés et al., 2014). In addition, overexpression of XBP1 has been shown to be protective against proteasome inhibition- or ischemic-induced cell death in neuronal cells (Sado et al., 2009; Ibuki et al., 2012). Likewise, in our *C. elegans* model for α -syn-induced DA neurodegeneration, we demonstrated that overexpression of XBP-1 is neuroprotective, whereas neuron-specific RNAi knockdown of *xbp-1* exacerbated the neurodegeneration.

These previous discoveries highlight the importance of identifying mediators of *xbp-1* activity, which take on added significance when considering neurodegeneration. Here we have shown that overexpression of RTCB-1 in DA neurons of worms expressing α -syn attenuated degenerating neurons from α -syn-induced toxicity, whereas neuron-specific depletion of *xbp-1* loses the protective function of RTCB-1 in the same strain of worms coexpressing RTCB-1 and α -syn in DA neurons. Similarly, we also showed that neuron-selective knockdown of *rtcb-1*

attenuates the neuroprotective phenotype of XBP-1 in worms expressing α -syn. These results uncover a functional relationship between RTCB-1 and XBP-1 in regulating neuroprotection against proteostatic stress caused by α -syn (Fig. 6). For the road ahead, an important goal is to explore the transcriptional status of RTCB-1 and XBP-1 in α -syn transgenic worms. Using qPCR and transgenic worms overexpressing α -syn in the nematode body wall muscles (Hamamichi et al., 2008), we have discerned that *rtcb-1* is not regulated by α -syn (data not shown). However, it is yet to be determined whether α -syn induces transcriptional activation of *xbp-1*. *In vitro* studies showed that α -syn oligomers induce *xbp-1* splicing (Castillo-Carranza et al., 2012), indicating that α -syn is an ER stressor. *In vivo* studies have also shown that XBP-1 significantly reduces α -syn levels (Si et al., 2012). Pathologically, our results support the idea that RTCB-1-induced activation of XBP-1 promotes clearance of abnormally accumulated α -syn and thus plays a neuroprotective role. Thus, identification of the signals modulating this cellular response may contribute to the treatment of PD.

Previously, an ER chaperone, Hsp70, was shown to inhibit α -syn-induced toxicity in a *Drosophila* PD model (Auluck et al., 2002). According to another study, mammalian Hsp70 interacts with IRE1, and this interaction prolongs the splicing of *XBP1*, thereby activating UPR target genes and protecting cells from cell death (Gupta et al., 2010). Likewise, we propose a model in which overexpression of RTCB-1 in *C. elegans* DA neurons might prolong the splicing of *xbp-1*, thus activating ER chaperone proteins and rescuing α -syn-induced neurodegeneration (Fig. 6), whereas cells depleted of RTCB-1 would hinder *xbp-1* splicing, thereby blocking UPR signaling and promoting cell death caused by α -syn (Figs. 2, 4). Recently, the mammalian RtcB has been identified as a UPR RNA ligase regulating XBP1 splicing *in vitro* (Lu et al., 2014). Moreover, cells expressing the ligase-dead RtcB (C122A) exhibited XBP-1 splicing defects. Our findings are consistent with these results in that *C. elegans* RTCB-1 also regulates XBP-1 splicing in the UPR pathway. We also determined that the ligase-active site of worm RTCB-1 (Cys¹²²) is necessary for its neuroprotective function. Our findings further extend the understanding of RTCB-1 function in the context of neurodegeneration and as a previously uncharacterized neuroprotective gene product with potential as a therapeutic target.

Growing evidence suggests that proteins functioning in RNA synthesis, processing, splicing, or degradation are mechanistically significant for neurodegenerative disorders (Cooper et al., 2009). Mutations in genes that are involved in RNA metabolism, such as TDP-43 (TAR DNA-binding protein) and FUS (fused in sarcoma, translocated in liposarcoma), have been found to cause motor neuron diseases (Gitcho et al., 2008; Vance et al., 2009). Both of these proteins are implicated in mRNA splicing (Belly et al., 2005; Buratti and Baralle, 2008). Additionally, mutations in tRNA splicing endonuclease (TSEN54) and tRNA synthetase have also been shown to cause motor neurodegeneration (Wan et al., 2012). The neuronal phenotypes caused by mutations in these genes could be a result of RNA processing defects as observed with RTCB-1 studies or could be attributable to alternate biochemical and cellular activities distinct from RNA processing (Antonellis et al., 2003; Jordanova et al., 2006; Latour et al., 2010). Interestingly, a recent *C. elegans* RNAi screen identified RTCB-1 (F16A11.2) as a modifier for GABAergic axonal regeneration (Nix et al., 2014). These and other studies suggest that defects in RNA metabolism (regulated at many different levels) can be pathogenically related to various neurodegenerative disorders. In this regard, an in-depth understanding of the pathways involving

RTCB-1, or its potential functional effectors, might yield promising and unexploited targets for therapeutic development to prevent neurodegeneration.

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