

Control of Rapsyn Stability by the CUL-3-containing E3 Ligase Complex^{*[S]}

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Seunghee Nam^{†1,2}, Kyoengwoo Min^{†1,3}, Hyejin Hwang[§], Hae-ock Lee[‡], Jung Hwa Lee[‡], Jongbok Yoon[¶], Hyunsook Lee[‡], Sungsu Park[§], and Junho Lee^{†4}

From the [†]Research Center for Cellulomics, Institute of Molecular Biology and Genetics, School of Biological Sciences, Seoul National University, 151-742 Seoul, Korea, the [§]Division of Nano Sciences (BK21), Ewha Womans University, 120-750 Seoul, Korea, [¶]Protein Network Research Center, Department of Biochemistry, Yonsei University, 134 Shinchon, 120-749 Seoul, Korea

Rapsyn is a postsynaptic protein required for clustering of nicotinic acetylcholine receptors (nAChRs) at the neuromuscular junction. Here we report the mechanism for posttranslational control of rapsyn protein stability. We confirmed that C18H9.7-encoded RPY-1 is a rapsyn homolog in *Caenorhabditis elegans* by showing that human rapsyn rescued *rpm-1* mutant phenotypes in nematodes, as determined by levamisole assays and micropost array behavioral assays. We found that RPY-1 was degraded in the absence of functional UNC-29, a non- α subunit of the receptor, in an allele-specific manner, but not in the absence of other receptor subunits. The cytoplasmic loop of UNC-29 was found to be critical for RPY-1 stability. Through RNA interference screening, we found that UBC-1, UBC-12, NEDD-8, and RBX-1 were required for degradation of RPY-1. We identified cullin (CUL)-3 as a component of E3 ligase and KEL-8 as the substrate adaptor of RPY-1. Mammalian rapsyn was ubiquitinated by the CUL3/KLHL8-containing E3 ligase *in vitro*, and the knockdown of KLHL-8, a mammalian KEL-8 homolog, inhibited rapsyn ubiquitination *in vivo*, implying evolutionary conservation of the rapsyn stability control machinery. *kel-8* suppression and *rpm-1* overexpression in *C. elegans* produced a phenotype similar to that of a loss-of-function mutation of *rpm-1*, suggesting that control of rapsyn abundance is important for proper function of the receptor. Our results suggest a link between the control of rapsyn abundance and congenital myasthenic syndromes.

Neuromuscular junctions, which are composed of a presynaptic terminal, basal lamina, and a postsynaptic terminal, are highly specialized structures through which neuronal signals are transmitted to muscles (1). Acetylcholine (ACh)⁵ is a major excitatory neurotransmitter at the neuromuscular junctions in various organisms, including nematodes and vertebrates (2–4). Nicotinic acetylcholine receptors (nAChRs), which consist of two α and three non- α subunits, cluster to facilitate ACh signaling at neuromuscular junction postsynaptic terminals (5). During cholinergic neuromuscular synaptogenesis in mammals, nAChRs cluster at the synapse; this activity is induced by neural agrin (6–9) and its postsynaptic receptor, muscle-specific kinase (MUSK) (10–13). Various cytoplasmic signaling proteins are involved in MUSK-mediated clustering of AChRs, including Abl (14), casein kinase 2 (15), β -catenin (16), adenomatous polyposis coli (17), and rapsyn (12, 18, 19). Rapsyn is essential for AChR clustering. Previous research demonstrated that coexpression of rapsyn and AChR, but not expression of AChR alone, in heterogeneous cells resulted in AChR clustering (18–21). Rapsyn was also shown to stabilize AChR clustering by binding to and inhibiting calpain, which plays a role in AChR cluster disassembly together with cyclin-dependent kinase 5 (22–24). A genetic study using mutant mice lacking rapsyn confirmed that it is essential for viability; the rapsyn knock-out mice died within hours after birth due to severe neuromuscular defects (12). In addition, it is well known that rapsyn is a causal gene for congenital myasthenic syndrome (CMS) (25, 26).

Control of rapsyn abundance has been known to be important for proper AChR clustering (27). Inhibition of AChR clustering by down-regulation or overexpression of rapsyn implies that tight regulation is necessary for homeostatic postsynaptic AChR clustering (28, 29). Such regulation can be accomplished by transcription factors, such as kaiso and β -catenin (30). The balance between positive and negative regulation of protein degradation also affects protein levels. The abundance of postsynaptic proteins, such as PSD-95, Shank, GKAP, AKAP79/150, and glutamate receptors is controlled by ubiquitination and proteasome-mediated turnover (31–35). Interestingly, a recent report suggested that the stability of rapsyn

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[S] The on-line version of this article (available at <http://www.jbc.org>) contains supplemental Figs. S1–S8, Tables S1–S3, and Movies 1–7.

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⁴ To whom correspondence should be addressed. Fax: 82-2-877-2661; E-mail: elegans@snu.ac.kr.

⁵ The abbreviations used are: ACh, acetylcholine; nAChR, nicotinic acetylcholine receptor; MUSK, muscle-specific kinase; CL, cytoplasmic loop; CMS, congenital myasthenic syndrome; RNAi, RNA interference; CUL-3, cullin 3; WT, wild type; GFP, green fluorescent protein; PI, proteasome inhibitor; HA, hemagglutinin; Ub, ubiquitin.

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can also be regulated at the posttranslational level (36). However, a detailed molecular mechanism for rapsyn regulation at the posttranslational level has not been elucidated.

In this report, we investigated rapsyn regulation at the posttranslational level using *Caenorhabditis elegans* as a model organism. Levamisole-sensitive nAChRs in the muscles of *C. elegans* are composed of LEV-1, UNC-29, UNC-38, and UNC-63, and resemble the mammalian nAChR in structure and function (2). Although no clear homolog of neural agrin or MUSK has been found (37, 38), *rpy-1* is a putative homolog of rapsyn. We first show that *rpy-1* of *C. elegans* is a structural and functional homolog of mammalian rapsyn. Through levamisole resistance assays and recently developed post-microassays, we show that *rpy-1* is required for AChR-mediated muscle function. We then elucidate a posttranslational mechanism for rapsyn stability control. We show that a functional reporter of muscular *C. elegans* RPY-1 is degraded by the ubiquitin-proteasome pathway in the absence of a non- α nAChR subunit, UNC-29. Through RNAi screening, we identified factors that are required for degradation of rapsyn. In particular, we identified cullin (CUL)-3 as a component of the E3 ligase complex and KEL-8 as the adaptor between CUL-3 and rapsyn; both proteins were conserved in mammals. We then show that the CUL-3-containing E3 ligase complex degraded rapsyn *in vitro*, and that knockdown of KLHL8, the mammalian KEL-8 homolog, inhibited ubiquitination of rapsyn in mammalian cells. Finally, we show that inhibition of RPY-1 degradation produced a phenotype that was similar to that seen with a loss-of-function mutation of the *rpy-1* gene. We conclude with a discussion of the potential link between rapsyn abundance and CMS.

EXPERIMENTAL PROCEDURES

Nematode Experiments—The *C. elegans* Bristol strain N2 was used as the WT strain (39). The N2 and mutant animals were grown at 20 °C on NGM plates. The *unc-29* (*e193*) and *rpy-1* (*ok145*) mutant strains were generous gifts from *Caenorhabditis* Genetics Center (Minneapolis, MN). The *rpy-1* (*ok145*) strain was backcrossed at least four times before being used in the experiments.

Assays for *rpy-1* Mutant Phenotypes—The effect of levamisole on muscle contraction and egg laying was examined. For the studies of muscle hypercontraction, adult animals were placed on 55-mm Petri dishes containing 50 μ M levamisole. After 4 h, the animals were observed for hypercontraction, and photographs were taken using a stereomicroscope. To measure the sensitivity of egg laying to levamisole, levamisole-treated animals were examined for the numbers of eggs that they laid over 3 h in the presence and absence of levamisole.

Microfabrication of Post-microarrays—We used photolithography (40) to prepare masters containing arrays of posts of SU-8 photoresist (MicroChem, Newton, MA) with 300- μ m diameter, 100- μ m height, and a distance of 150 μ m between the posts (41). The actual masters that were used for molding of the agar (positives) were replicas (negatives) of these photolithographically prepared masters in Sylgard 184 polydimethylsiloxane (Dow-Corning, Midland, MI). Bacto-agar (4%) in NGM buffer (50 mM NaCl, 1 mM CaCl₂, 1 mM MgSO₄, and 25 mM KH₂PO₄) was poured at 70 °C onto the polydimethylsilox-

ane mold in a Petri dish and left to cool at room temperature. Air bubbles trapped in the small features of the mold were released by scraping the surface of the agar with the edge of a razor blade. After 1 h, the agar was cut and lifted from the mold and placed structure-side up onto a Petri dish. Just before use, the agar grid structure was filled with NGM buffer. L4 and young adult worms were selected from growth plates with a platinum worm pick, washed in NGM buffer, and placed into the fluid-filled agar microstructure. Worms were confined to the structure by the surface tension of the liquid layer. NGM buffer was added to the structure to offset evaporation.

Data Collection from Post-microarray Experiments—Movies of worms moving in the microstructures were taken using an SMZ 1500 stereomicroscope (Nikon Instruments, Japan) with a CCD camera (DS-Fi1; Nikon Instruments, Japan). The motion of the worms was analyzed using ImageJ (National Health Institutes, Bethesda, MD).

Construction of the Reporter Genes and *rpy-1* Derivatives—For examination of *rpy-1* expression patterns, we constructed a reporter gene that encoded the full-length *C. elegans rpy-1* gene fused to GFP and *Discosoma* sp. red reporter genes. To ensure avoidance of mosaicism, which is often caused by extrachromosomal arrays, we observed GFP patterns in at least five independent transgenic lines. To examine the role of the RING domain of RPY-1, we created a construct that contained a mutated RING domain in which two His histidine residues were changed to glutamines by an overlap PCR method using the indicated primer sets. PCR was first performed using primer sets 43-5/43-7 and 43-6/43-8; a second round of PCR was performed with the 43-5 and 43-8 primers and the initial PCR products as template. This mutant *rpy-1* gene was then subcloned into the pPD95.75 (pJL525) plasmid. The presence of the mutations in pJL525 was confirmed by sequencing. To examine whether the human RAPSN gene could rescue the *rpy-1* (*ok145*) mutation, we designed constructs in which the cDNAs of the representative RAPSN isoform (NM_032645) were fused to the GFP reporter gene under the control of a muscle-specific promoter, *myo-3*. As a positive control, we designed a construct that contained the *C. elegans rpy-1* cDNA fused to the GFP reporter gene. Each cDNA was amplified by PCR using the primer sets 43-132/43-133 (for *C. elegans rpy-1* cDNA) and hRAPSN-1/hRAPSN-2 (for human RAPSN cDNA) and subcloned into the *Pmyo-3::gfp* vector (modified pPD114.108). The constructs were confirmed by sequencing. The sequences of the primers are listed in supplemental Table S3. All reporter vectors were a gift from Dr. Andrew Fire (Stanford University, CA).

The Construction of *unc-29* Reporter Genes and Chimeric Derivatives—To examine the relationship between RPY-1 and UNC-29, we constructed the full-length *C. elegans unc-29* gene by PCR with primer sets *unc-29-9/unc-29-10*. To test the role of the cytoplasmic loop region of UNC-29, we created a construct with this region deleted (*unc-29* Δ CL), as well as constructs with deletions of transmembrane domain 1, 2 (*unc-29* Δ M1, 2), by PCR using the primer sets *unc-29-29/unc-29-37*, *unc-29-38/unc-29-33*, *unc-29-9/unc-29-13*, and *unc-29-14/unc-29-10*, respectively. We made *unc-29* chimeric constructs in which each cytoplasmic loop of UNC-38, UNC-63, and

LEV-1 was independently fused to the *unc-29* Δ CL construct. These constructs were confirmed by sequencing.

Germ Line Transformation—Germ line transformations were carried out using standard protocols (42). The coinjection marker used was the dominant *rol-6* DNA pRF4 and *sur-5* gene. At least three independent lines were established and used for experiments.

Proteasome Inhibitor Treatment and Analysis of RPY-1 in *C. elegans*—To test whether RPY-1 is degraded by the proteasome pathway, we treated the animals with a commonly used proteasome inhibitor mixture (PI) containing MG132 and lactacystin (Cayman Chemical, Ann Arbor, MI). The PI experiments were performed as previously described, with some modifications (43–45). Briefly, the wild-type and *unc-29* mutant animals containing the *rpm-1::gfp* reporter construct were grown in liquid culture (wormbook.org/chapters/www_strainmaintain), purified by sucrose flotation, and collected into a 15-ml tube. PI was added at a final concentration of 50 μ M, after which the animals were incubated for 6 h at 20 °C on a Nutator mixer before being washed with M9. To extract protein from PI-treated animals, collected animals were frozen at –80 °C and then ground in liquid nitrogen and resuspended in lysis buffer (50 mM HEPES/KOH (pH 7.6), 1 mM EDTA, 150 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, 1 mM PMSF, protease inhibitor mixture (Calbiochem, Darmstadt, Germany), 10 mM *N*-ethylmaleimide (Sigma)). This lysis buffer was mixed as described (wormbook.org/chapters/www_intromethodscellbiology), with some modifications. The extract was centrifuged at 18,000 \times *g* for 1 h and pre-cleared with protein G-agarose (Upstate, Charlottesville, VA) for 1–2 h at 4 °C and then incubated with anti-GFP tag-agarose (MBL, Nagoya, Japan) overnight at 4 °C. After washing with the wash buffer (50 mM HEPES/KOH, pH 7.6, 1 mM EDTA, 150 mM NaCl, 0.1% Nonidet P-40, 1 mM dithiothreitol, 1 mM PMSF, protease inhibitor mixture), bound proteins were eluted in the reducing SDS-PAGE sample buffer. Samples were then centrifuged briefly and the supernatant loaded onto a 6% SDS-PAGE gel. The proteins were transferred onto a nitrocellulose transfer membrane. The blots were first incubated with primary antibodies for 1 h and then with the secondary antibodies (horseradish peroxidase-conjugated anti-rabbit antibodies, diluted 1:5000; Amersham Biosciences) for 40 min in TBST. The RPY-1::GFP proteins were detected using an anti-RPY-1 antibody. An anti-actin antibody was used as a loading control.

RNAi Experiments—To identify genes responsible for rapsyn degradation by the proteasome pathway, we performed RNAi experiments. We investigated all available UBC genes by using the previously described feeding method (46). We used an RNAi library purchased from MRC (Cambridge, UK). In addition, we created the *ubc-3*, *cul-2*, and *cul-3* constructs for RNAi because these genes were not covered by the library. The *cul-2*, *cul-3*, and *ubc-3* cDNAs were amplified by reverse transcriptase-PCR using primer sets *cul-2-1/cul-2-2*, *cul-3-1/cul-3-2*, and *ubc-3-1/ubc-3-2*, respectively, and subcloned into an L4440 feeding vector (pPD129.36). These constructs were confirmed by sequencing. We transformed the obtained constructs into HT115 (DE3) bacterial cells and performed RNAi feeding. For genes whose RNAi-mediated knockdown caused embryonic lethality, we fed L1 larvae with RNAi bacteria and observed

the phenotypes when they reached adulthood. The sequences of the primers are listed in supplemental Table S3.

Yeast Two-hybrid Assay—To determine whether rapsyn, KEL-8, and CUL-3 interact with one another, we performed yeast two-hybrid assays. The bait plasmids, *cul-1::pAS2-1*, *cul-3::pAS2-1*, WT *rpm-1::pAS2-1*, and mRING *rpm-1::pAS2-1*, were constructed by subcloning cDNAs amplified by PCR using the primer sets *cul-1-1/cul-1-2*, *cul-3-3/cul-3-2*, and 43-114/43-18, respectively, into the pAS2-1 vector. We designed a prey plasmid (*kel-8::pACTII*) by subcloning *kel-8* cDNA, which was amplified by reverse transcriptase-PCR using the primer set *kel-8-10/kel-11*, into the pACTII vector. These constructs were confirmed by sequencing. Each construct was transformed into yeast strain cells (Clontech, Mountain View, CA), which were then cultured on Trp[–]Leu[–] or Trp[–]Leu[–]His[–] plates. The sequences of the primers are listed in supplemental Table S3.

Antibody Production—The *rpm-1* cDNA fragment encoding amino acids 453–597 of the RPY-1 protein was amplified by PCR using primer set 43-152/153 and then inserted into pRSETA using BamHI and HindIII (Invitrogen). This recombinant His₆::RPY-1 protein was expressed in BL21 *Escherichia coli* and purified according to the QIAexpressionist protocol (Qiagen). Antibody was produced by PEPTRON Inc. (Daejeon, Korea).

Cell Culture Experiments—We purchased human rapsyn cDNA from Invitrogen. Full-length cDNAs of KLHL8 (KIAA1328) and KEAP1 (KIAA0132) were generously provided by the Kazusa DNA Research Institute. To construct plasmids for the expression of the N-terminal FLAG-, MYC-, or HA-tagged proteins, cDNAs were amplified by PCR with the appropriate primers and ligated into the pcDNA3.1(+) vector (Invitrogen). For expression of C-terminal FLAG-tagged proteins, the PCR products, which were generated with appropriate primers, were ligated into the p3XFLAG-CMV-14 vector (Sigma). The constructs for the expression of CUL3 and RBX1 were previously described (47). All protein processing was performed in accordance with standard protocols.

ts20 Cell Experiments—ts20 cells were transfected with MYC::RPY-1 at 34 °C. 24 h later, cells were split and maintained at either 34 or 40 °C for an additional 12 h, and then treated with cycloheximide (20 μ g/ml). Cells were harvested at the indicated time points and Western blot analysis was performed for anti-MYC and anti-actin monoclonal antibody. The relative levels of MYC::RPY-1 and actin were obtained by densitometry using Multi Gause version 3.0 (Fujifilm). MYC::RPY-1/actin levels were arbitrarily set as 1 for the 0-h samples for each experimental group.

In Vitro Ubiquitination—The constructs expressing HA-CUL3, HA-RBX1, and MYC-KLHL8 (or MYC-KEAP1) were co-transfected into a HeLa cell line. MYC-KLHL8 is a chimeric gene that contains the BTB domain of KEAP1 and the Kelch domains of KLHL8. This was done because the full-length KLHL8 protein was difficult to obtain as a soluble protein. The HA-CUL3/RBX1/MYC-KLHL8 complex (E3) was purified using HA-conjugated agarose beads (Sigma). Recombinant FLAG-RAPSYN was expressed in SF21 cells and purified by Newgex Inc. (Seoul, Korea). The Ub ligation reaction mixtures

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were incubated at 37 °C for 1 h and proteins were analyzed by Western blotting.

Short Hairpin RNA Experiments—The short hairpin RNA expression lentiviral vectors used for targeting of the KLHL8 gene and a control vector were purchased from Macrogen Inc. (Seoul, Korea). 3T3 cells were infected with 35 ml of 10-fold concentrated virus for 8 h and then incubated for a further 48 h. Stable lines expressing short hairpin-red fluorescent protein were selected and used for further experiments.

RESULTS

RPY-1 Is a Homolog of Rapsyn in *C. elegans*—The *C. elegans* RPY-1 protein, a putative rapsyn homolog encoded by C18H9.7, contains eight TPR domains and a RING domain. These domains are conserved in rapsyn proteins of other species (supplemental Fig. S1, A and C). We first examined the phenotype of a deletion mutation in the *rpy-1* gene. The examined mutation was *rpy-1 (ok145)*, which contains a deletion of exons 4 through 10 that results in a severely truncated protein (supplemental Fig. S1B). Similar to animals that carry mutations in nAChR genes, *rpy-1 (ok145)* mutant animals were resistant to levamisole, an agonist of nAChRs (48, 49); they did not hypercontract or show increased egg laying after treatment (Fig. 1, A–C). Although *rpy-1* mutant animals clearly showed levamisole resistance typical of nAChR mutations, they did not show significant defective motility on media plates in the absence of levamisole treatment (Fig. 1D). For increased sensitivity, we applied the post-microarray assay, which is a recently developed sensitive behavioral assay (41). With this method, we were able to detect a significant motility defect in *rpy-1 (ok145)* mutant animals (Fig. 1D). We then tested whether a GFP reporter gene harboring the *rpy-1* gene (pJL524) could rescue *rpy-1* mutation phenotypic characteristics. Muscle hypercontraction and increased egg laying caused by this mutation were rescued by injection of the *rpy-1::gfp* construct at a concentration of 75 μ g/ml (Fig. 1, A–C; supplemental Table S1). The motility defect detected by the post-microarray assay was also rescued (Fig. 1D, supplemental Movies S1–S7). We then found that the expression of *rpy-1* in muscles using the *myo-3* promoter rescued the *rpy-1* mutant phenotypes (Fig. 1, A–C; supplemental Table S1). Furthermore, we found that expression of the human rapsyn gene in the muscles of the nematode *rpy-1* mutant rescued levamisole resistance (Fig. 1, A–C, supplemental Table S2) and motility defects (Fig. 1D, supplemental Movie S7). Although it is formally possible that RPY-1 and mammalian rapsyn may have distinct functions, the most probable interpretation of the data presented above is that RPY-1 is the functional homolog of rapsyn in the nematode.

RPY-1 Is Expressed in Muscles and Neurons—Next, we examined the expression pattern of *rpy-1* using a reporter gene construct that contained the putative promoter region and full-length coding region of *rpy-1*. RPY-1 was first detected at 260–270 min in embryonic development (data not shown) and persisted throughout development (Fig. 2A). In adult animals, RPY-1 was expressed in body-wall muscles, head muscles, and sex-specific muscles of hermaphrodites. The *rpy-1::gfp* reporter gene was also expressed in neurons (Fig. 2A).

Muscular RPY-1 Is Destabilized in the Absence of UNC-29, but Not Other nAChR Subunits—Knowing that *rpy-1* is a rapsyn homolog, we started to investigate the unsolved question of whether and how RPY-1 stability is controlled. Proteins that are physically associated with rapsyn *in vivo* are good candidates for effectors of RPY-1 protein stability control. To evaluate whether RPY-1 protein abundance is dependent on the presence of its binding partner, nAChR, we examined whether the steady-state levels of RPY-1::GFP were influenced by the absence of functional nAChR subunits. We examined *rpy-1::gfp* expression in animals whose muscle nAChR subunit genes (*unc-29*, *unc-38*, *unc-63*, and *lev-1*) were individually knocked down by RNAi. We found that the steady-state level of RPY-1::GFP in muscle cells was reduced by RNAi targeting *unc-29* but not by RNAi against the other nAChR subunits (Fig. 2, B and C). We obtained identical results in experiments in which we examined the steady-state levels of RPY-1 in reference mutant strains for each subunit (Fig. 2, B and C). The transcript level of *rpy-1* in the *unc-29 (e193)* mutant worms was not reduced but rather increased probably due to compensatory effects (supplemental Fig. S2), suggesting that destabilization of RPY-1 is not due to the decrease in the *rpy-1* transcription in muscles. Taken together, these results indicate that UNC-29, but not the other nAChR subunits, is required for stable maintenance of RPY-1 proteins.

Allele-specific Requirement of UNC-29 for Rapsyn Stability—We expressed the *rpy-1::gfp* reporter gene (pJL524) in various *unc-29* mutant backgrounds to further evaluate the role of UNC-29 in control of RPY-1 stability. We examined the *e193*, *e522*, and *x30* alleles (supplemental Fig. S3). The *unc-29 (e193)* mutation is a missense mutation of proline (Pro) to serine (Ser) at an evolutionarily conserved residue within the M2 transmembrane domain, which forms the inner channel gate of the receptor (50–52). The *unc-29 (e522)* mutation is a missense mutation of glycine (Gly) to glutamic acid (Glu) within the M4 transmembrane domain of the receptor, and the *unc-29 (x30)* mutation is a missense mutation of glutamic acid (Glu) to lysine (Lys) within the extracellular domain of the receptor. Although the expression of RPY-1::GFP was significantly reduced in the muscles of *unc-29 (e193)* mutant animals (Fig. 2B), RPY-1::GFP was not degraded in the *unc-29 (e522)* or *unc-29 (x30)* mutant backgrounds (supplemental Fig. S4). These results suggest that stable expression of RPY-1 in muscles requires a specific assemblage of functional UNC-29 proteins and that specific amino acids located within the extracellular transmembrane domains or the M4 transmembrane domain may not be critical for the regulation of rapsyn stability.

The Cytoplasmic Loop of UNC-29 Is Crucial for RPY-1 Stability—Each subunit of nAChRs contains long cytoplasmic loops (CLs) between the third and fourth transmembrane domains, raising the possibility that the CL domain of UNC-29 is the RPY-1 binding domain. We found that expression of a *unc-29* construct that lacks the CL domain was unable to stabilize RPY-1 in *unc-29* mutant animals (Fig. 2D). When the first two transmembrane domains of UNC-29 were deleted, leaving the CL domain intact, RPY-1 stability was restored (Fig. 2E), strongly suggesting that the CL domain is responsible for binding to RPY-1. To closely examine the specific interactions

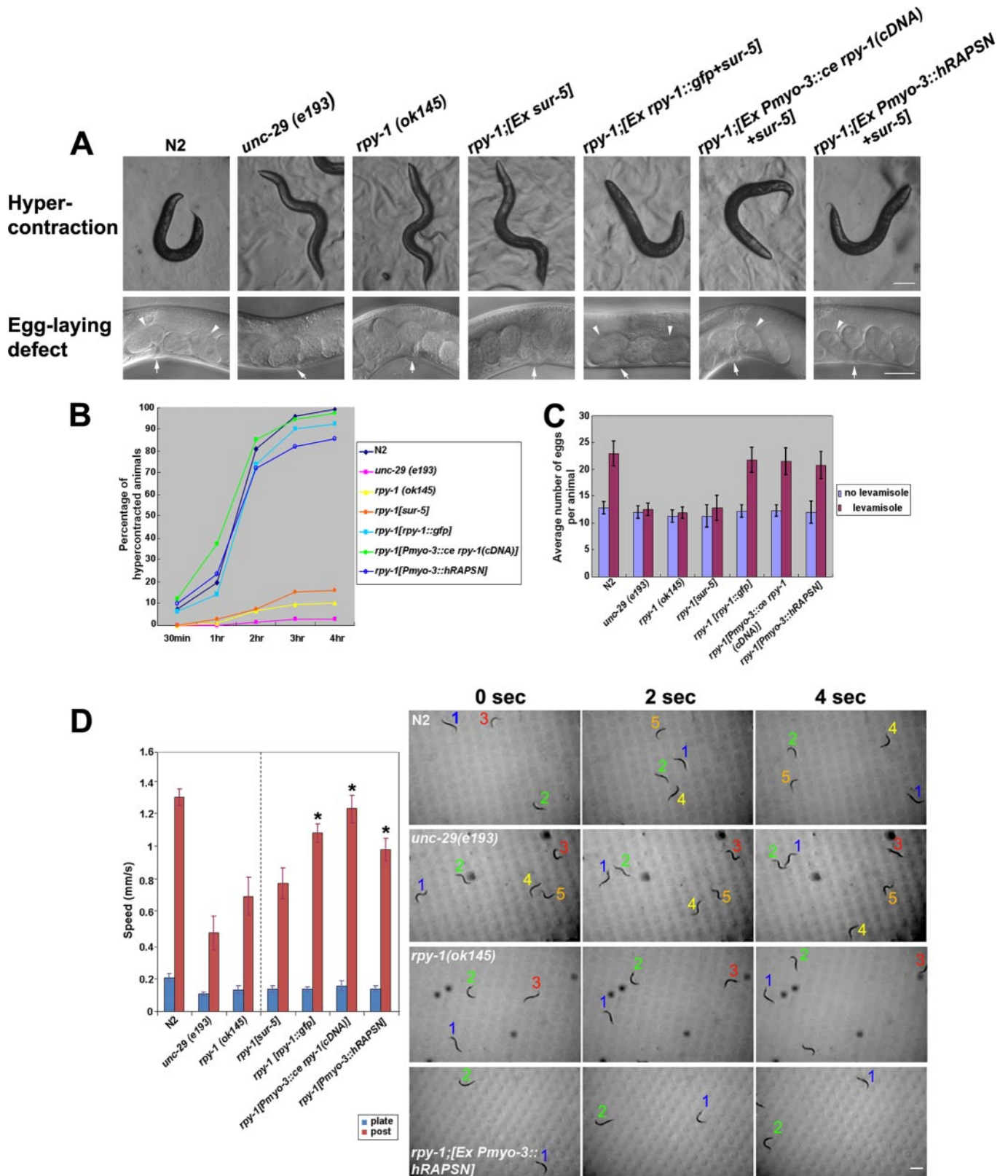


FIGURE 1. Analysis of *rpy-1* mutant phenotype in *C. elegans*. A, the RPY-1::GFP reporter was functional *in vivo*. The *rpy-1* mutant phenotypes were rescued by the expression of the *rpy-1::gfp* reporter or the human rapsyn gene under the control of both the *rpy-1* promoter and muscle-specific promoter (*Pmyo-3*) in the levamisole assay. The upper panels show muscle hypercontraction, and the bottom panels show enhanced egg laying. The scale bars represent 100 (top row) and 50 μm (bottom row), respectively. Anterior is on the left and dorsal at the top. The arrows indicate the locations of the vulvae, and the arrowheads indicate two-cell stage eggs. B and C, the graphs summarize the results of the levamisole assay. B represents hypercontraction and C represents the egg-laying defect observed in A. D, the rescue of the *rpy-1* mutant phenotypes by the *rpy-1* expression using the *rpy-1* promoter and the *myo-3* promoter as analyzed by the micropost assay. The left graph represents post-levamisole assay results and the right panels show the movements of the strains. *, $p < 0.001$. The numbers indicate individual worms. The scale bar represents 500 μm .

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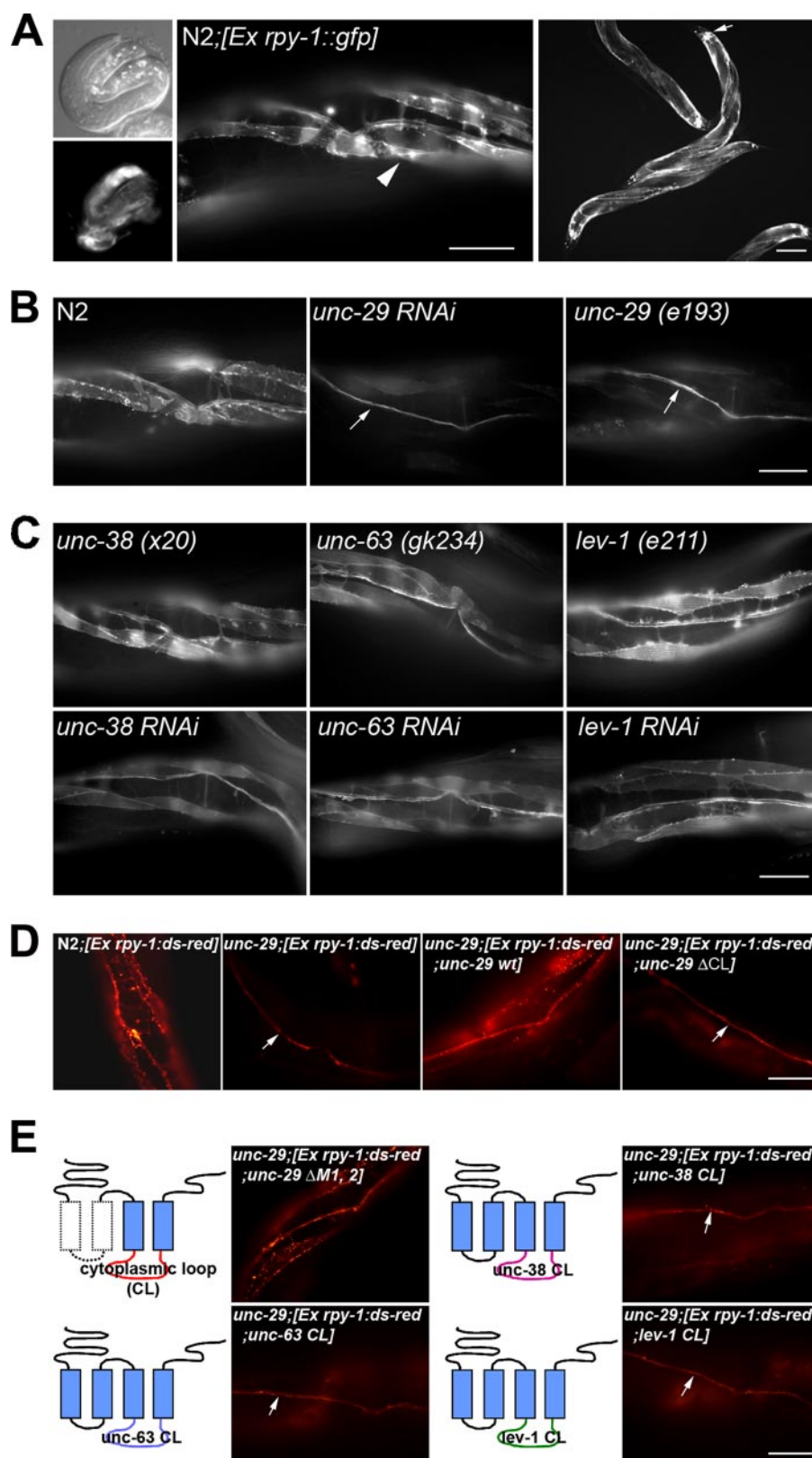


FIGURE 2. The RPY-1 protein was unstable in the absence of UNC-29. *A*, the RPY-1 protein is expressed in muscles and neurons. The *left panels* show a 3-fold stage embryo; the *middle and right panels* are adults. The *scale bars* indicate 50 (*middle image*) and 100 μm (*right panel*). *B*, expression patterns of the RPY-1::GFP in N2, *unc-29* RNAi, and mutant backgrounds. The *arrows* indicate the ventral nerve cord (VC). The *scale bar* represents 50 μm . *C*, GFP expression patterns of the RPY-1 protein in other nAChR subunit genes, *unc-38*, *unc-63*, and *lev-1*, RNAi, and mutant backgrounds, as indicated. The *scale bar* represents 50 μm . *D*, RPY-1 protein stability was dependent on the presence of the UNC-29 protein but not on the other nAChR subunits, UNC-38, UNC-63, or LEV-1 proteins. The *arrows* indicate the VC. The *scale bar* represents 50 μm . *E*, specific requirement of the cytoplasmic loop domain of UNC-29 for RPY-1 stability. The *upper left panels* show the effect of the deletion of two transmembrane domains of UNC-29 on the stability of RPY-1. The other panels show the effects of chimeric UNC-29 proteins, each of which contained the CL domain of UNC-38, UNC-63, or LEV-1, on the stability of RPY-1. The *arrows* indicate the VC. The *scale bar* represents 50 μm .

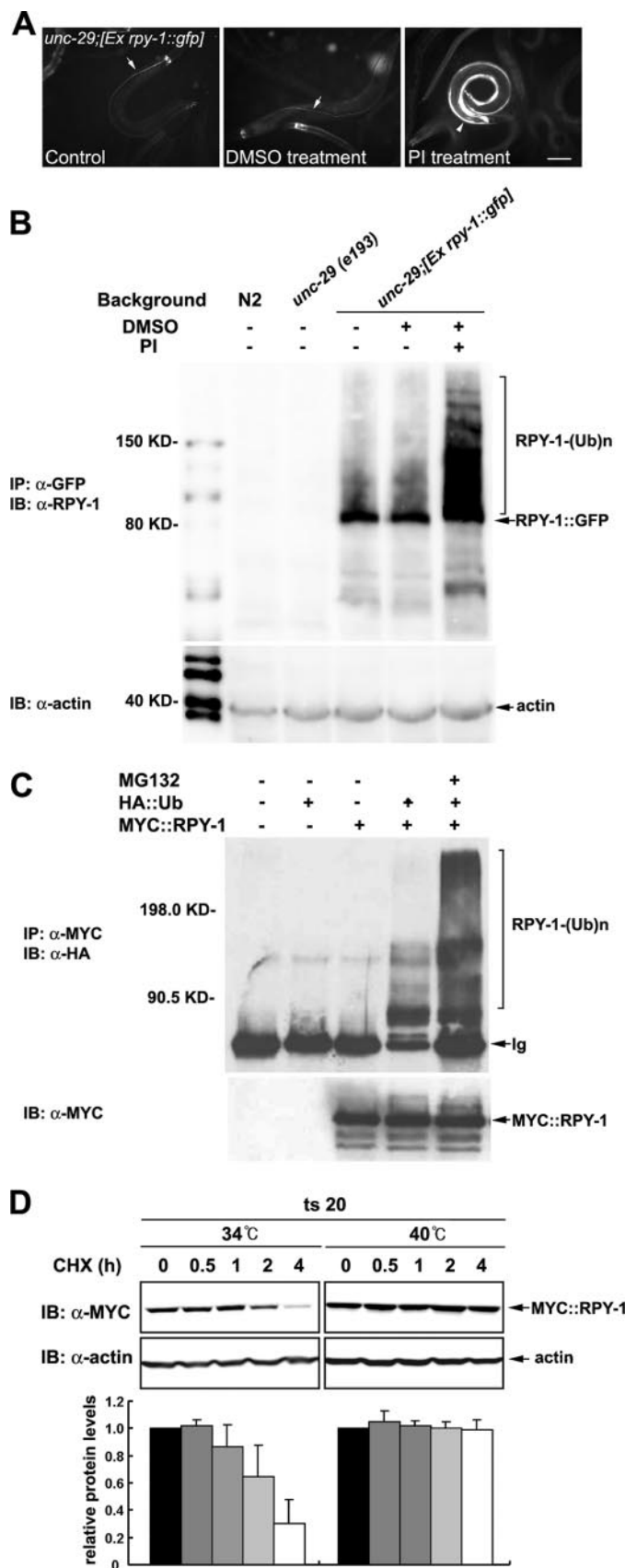


FIGURE 3. RPY-1 is degraded by the ubiquitin-proteasome pathway. *A*, the RPY-1::GFP protein was stabilized *in vivo* by treatment with a proteasome inhibitor mixture (PI). The RPY-1::GFP protein is in the *unc-29* background. The arrows indicate the ventral nerve cord. The scale bar represents

between the RPY-1 and UNC-29 CL domains, we constructed chimeric UNC-29 constructs, each of which contained the CL domain of UNC-38, UNC-63, or LEV-1. None of these constructs restored RPY-1 stability in the *unc-29* mutant background (Fig. 2*E*), indicating that the CL domain of UNC-29 is indeed critical for RPY-1 stabilization.

RPY-1 Is Degraded by the Ubiquitin-proteasome Pathway—Next, we examined whether the ubiquitin-proteasome pathway is involved in the degradation of RPY-1. We found that *unc-29* (*e193*) mutant animals containing the RPY-1::GFP transgene showed higher RPY-1 GFP expression in muscles after treatment with PI (PI mixture: MG132 and lactacystin) without increasing the transcript level of the gene (Fig. 3*A*, Fig. S2). Consistent with this finding, polyubiquitinated RPY-1 proteins were visible by Western blot analysis of anti-GFP immunoprecipitates of PI-treated animals (Fig. 3*B*). To evaluate the post-translational control of RPY-1 under normal conditions, we examined the degradation of RPY-1 in wild-type animals injected with 25 $\mu\text{g}/\text{ml}$ RPY-1 GFP transgene, which was one-third of the concentration used in the rescue experiment described above. The expression of the RPY-1::GFP was substantially lower in these transgenic animals than in those injected with the concentration of 75 $\mu\text{g}/\text{ml}$ of the transgene (supplemental Fig. S8). The transgenic animals were as sensitive to levamisole as nontransgenic WT animals (data not shown). We found that RPY-1::GFP was highly stabilized after PI treatment (supplemental Fig. S5). These results indicate that RPY-1 is ubiquitinated and degraded by the proteasome pathway not only in the absence of a functional UNC-29 protein but also in the wild-type background. We then examined whether nematode RPY-1 proteins are ubiquitinated in mammalian cells by expressing nematode RPY-1 in 293T cells. Fig. 3*C* shows that addition of Ub and MG132 greatly enhanced the ubiquitination of RPY-1 in these cells. In addition, we found that hamster ts20 cells containing a temperature-sensitive E1 mutation (53) could not degrade RPY-1 at a nonpermissive temperature (Fig. 3*D*). RPY-1 was efficiently degraded at the permissive temperature with a half-life of about 4 h and was not degraded after 4 h at the nonpermissive temperature (Fig. 3*D*). Of note, the overall level of MYC::RPY-1 expression was higher for the cells maintained at the nonpermissive temperature compared with that at the permissive temperature. These results suggest that rapsyn and its degradation machinery are evolutionarily conserved. Indeed, as shown below, all proteins

100 μm . *B*, PI treatment blocked RPY-1 protein degradation in the *unc-29* mutant animals. A small amount of the RPY-1::GFP protein was present without PI treatment, probably due to its persistent expression in neurons (lanes 4 and 5). When treated with the PI, polyubiquitinated RPY-1::GFP proteins were visible (lane 6). The RPY-1::GFP protein was detected by Western blot analysis with anti-RPY-1 antibody. Western blot analysis using an anti-actin antibody was used as a loading control. *C*, RPY-1 was ubiquitinated in cultured cells. 293T cells were cotransfected with MYC-tagged WT RPY-1 and HA-tagged Ub. After 48 h, the cells were treated with 20 μM MG132 for 6 h (lane 5) or left untreated. RPY-1-Ub complexes were analyzed by immunoprecipitation (IP) with an anti-MYC antibody (9E10) followed by immunoblotting (IB) using monoclonal anti-HA antibodies. The same blot was reprobed with 9E10 to normalize RPY-1 immunoprecipitation. *D*, RPY-1 degradation depends on the ubiquitin pathway. The upper panels show representative results. The bottom graphs summarize the results of ts20 cell experiments. Each bar represents average value \pm S.E. from 3 independent experiments. Actin was used as a loading control. CHX, cycloheximide; DMSO, dimethyl sulfoxide.

Stability Control of Rapsyn

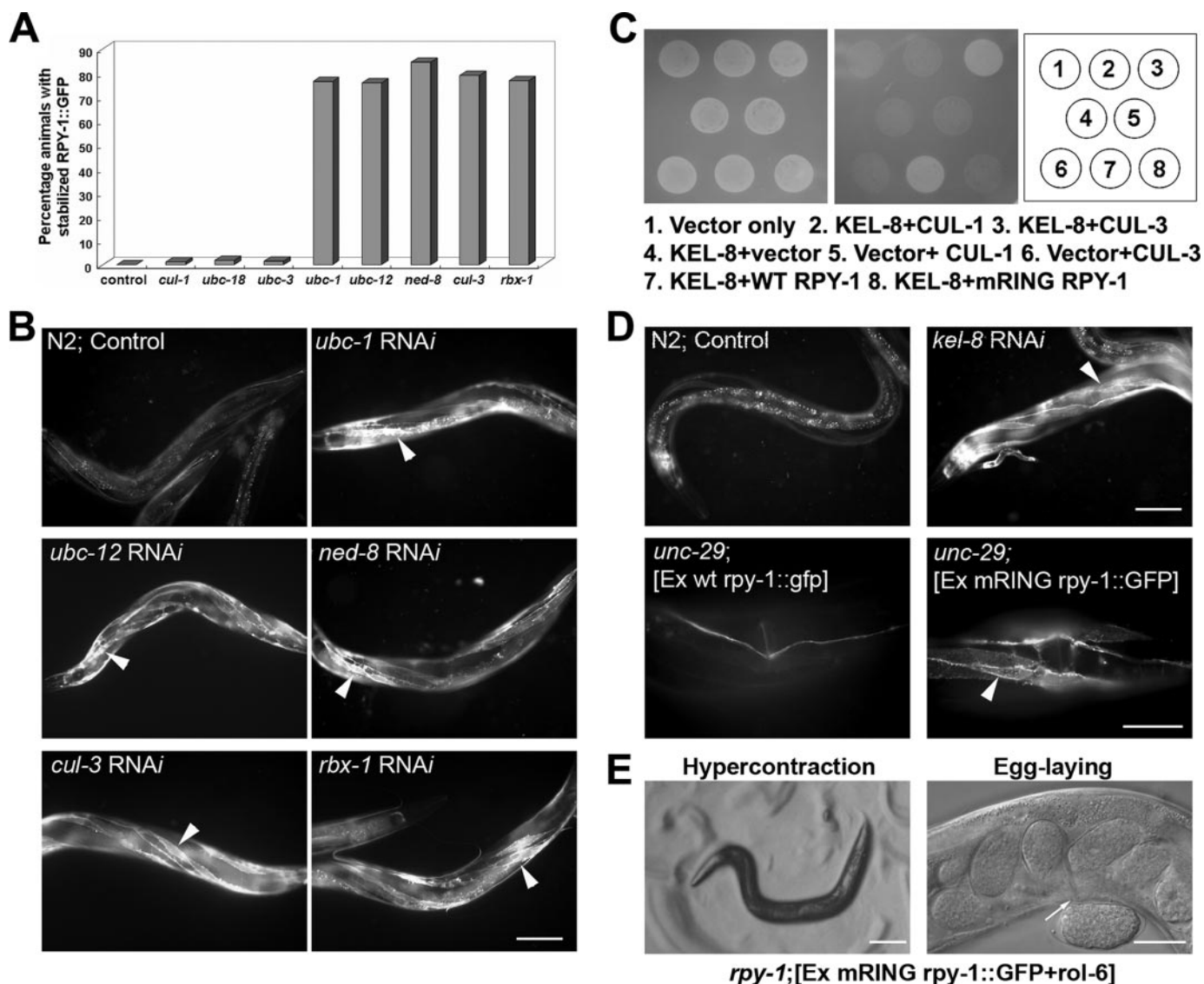


FIGURE 4. The identification of factors involved in RPY-1 degradation. *A* and *B*, identification of UBC-1, UBC-12, NED-8, RBX-1, and CUL-3. The graph (*A*) summarizes the results of RNAi assays with genes involved in the degradation of RPY-1. The y axis represents the percentage of animals with stabilized RPY-1::GFP in the muscles after each RNAi experiment. *B*, the panels show representative effects of RNAi on RPY-1 stability in the wild-type background. The arrowheads indicate stable RPY-1::GFP expression in the muscles. The scale bar represents 100 μ m. *C* and *D*, KEL-8 is the substrate adaptor for RPY-1 ubiquitination. *C*, the physical interaction of KEL-8 with rapsyn and CUL-3 as determined by yeast two-hybrid assays. KEL-8 interacts with CUL-3 and RPY-1. KEL-8 did not interact with RPY-1[mRING, H395, 398Q-RING]::GFP, which carries two His to Gln mutations in the RING domain. *D*, the degradation of RPY-1 required KEL-8 and the RPY-1 RING domain. The upper panels show the expression of RPY-1::GFP in wild-type animals with or without *kel-8* RNAi. The arrowhead indicates the expression of RPY-1 GFP in the muscles. The scale bar represents 100 μ m. The bottom panels show *unc-29* (*e193*) animals carrying either a wild-type *rpy-1* transgene (left panel) or the *rpy-1*[mRING]::gfp reporter (right panel). The arrowhead indicates the muscles. The scale bar represents 25 μ m. *E*, the RING domain was required for the function of RPY-1. The left panel shows that muscle hypercontraction was absent after levamisole treatment; the right panel shows that enhanced egg laying was absent after levamisole treatment. The scale bars represent 100 (left panel) and 25 μ m (right panel).

involved in rapsyn degradation have been well conserved throughout evolution.

Identification of *ubc-1*, *ubc-12*, *nedd-8*, *rbx-1*, and *cul-3* as Factors Required for Rapsyn Degradation—To identify protein factors responsible for rapsyn degradation, we performed RNAi experiments in which we knocked down each gene that had been previously linked to ubiquitination. Effects on RPY-1 degradation in wild-type animals were studied by analysis of animals following injection of the transgene (25 μ g/ml). We then knocked down most of the E2 enzyme genes in *C. elegans* and found that knockdown of *ubc-1* and *ubc-12* resulted in up-regulation of RPY-1 (Fig. 4, *A* and *B*; supplemental Fig. S6). The

involvement of UBC-12, an E2 enzyme for neddylation, raised the possibility of the involvement of a cullin-E3 complex, because cullin complexes are E3-Ub ligase complexes that undergo neddylation (54, 55). We subsequently found that RNAi-mediated suppression of *nedd-8*, *rbx-1*, and *cul-3*, but not other cullin genes, stabilized RPY-1 (Fig. 4, *A* and *B*, supplemental Fig. S6). These RNAi results strongly suggest that the CUL-3-containing protein complex is the E3 ligase required for RPY-1 ubiquitination.

Identification of *KEL-8* as a Substrate Adaptor That Binds Rapsyn—To identify the adaptor protein that links E3 ligase and RPY-1, we searched for proteins that had previously been

shown to physically interact with RPY-1. We found that KEL-8, encoded by W02G9.2, was a strong candidate as it was previously identified as an interaction partner of RPY-1 in a yeast two-hybrid screening assay (56) and shown to contain a BTB/POZ domain and six Kelch motifs. BTB/POZ domain proteins are known to directly interact with CUL3 and several proteins containing both BTB/POZ domain and six Kelch motif are substrate adapters for CUL3-containing E3 ubiquitin ligase complex (57–62). We confirmed that KEL-8 interacted with the N-terminal domain of CUL-3 as well as with the full-length RPY-1 protein in yeast two-hybrid assays (Fig. 4C). Consistent with KEL-8 acting as an adaptor between the CUL-3 complex and RPY-1, we found that *kel-8* RNAi stabilized RPY-1::GFP (Fig. 4D). We also found that an RPY-1 protein with a mutant RING domain failed to interact with KEL-8, indicating that the RING domain is required for the interaction (Fig. 4C). However, the RING domain itself was not sufficient for binding to KEL-8 (data not shown). In addition, we found that RPY-1 proteins with a defective RING domain were stabilized, even in the *unc-29* (*e193*) mutant background (Fig. 4D), but that the *rpm-1::gfp* reporter gene with the defective RING domain did not rescue the *rpm-1* mutant phenotype (Fig. 4E).

Proteins Involved in Rapsyn Degradation Are Conserved in Mammals—We noticed that all components required for RPY-1 degradation in the nematode are conserved in mammals. For example, KEL-8 is homologous to KLHL8 in humans (supplemental Fig. S7). To examine the evolutionary conservation of the physical interaction between the KEL-8 adaptor and rapsyn, we tested whether KLHL8 could bind to mammalian rapsyn. We found that KLHL8 was able to bind to human rapsyn and CUL3 by co-immunoprecipitation and Western blot analysis (Fig. 5A, lane 2). As a negative control, KEAP1, a protein that contains BTB and Kelch domains (59, 60), did not bind to rapsyn (Fig. 5A, lane 1). We then mapped the internal domain of KLHL8 for its interaction with CUL3 or rapsyn. For mapping, we generated four additional constructs that encode FLAG-tagged KLHL8 fragments (described in the figure legend) and co-expressed them with HA-tagged CUL3 or Myc-tagged rapsyn. We found that KLHL8 interacted with CUL3 through its BTB domain and bound to rapsyn via its Kelch domain (Fig. 5A, lanes 3–6). Next, we performed *in vitro* ubiquitination assays using recombinant human rapsyn, yeast Uba1 (E1), human UbcH5A (E2), Ub, and the immunoprecipitated KLHL8/CUL3-containing E3 ligase complex (Fig. 5B). The KEAP1/CUL3 complex was used as a negative control. We found that rapsyn was polyubiquitinated by KLHL8-containing E3 ligase, but not by KEAP1-containing E3 ligase, clearly indicating that rapsyn is a direct substrate of KLHL8-containing E3 ligase in mammals. We next examined the effect of KLHL-8 depletion on the ubiquitination of rapsyn by performing RNAi experiments in mammalian cells. We found that knockdown of KLHL8 in 3T3 cells reduced the level of rapsyn ubiquitination (Fig. 5C), again indicating that the maintenance mechanism for rapsyn stability is conserved in mammals.

RPY-1 Accumulation by *kel-8* Suppression or *rpm-1* Overexpression Resembles the *rpm-1* Mutation—To examine the physiological consequences of RPY-1 accumulation following KEL-8 depletion, we examined the phenotypes of *kel-8* knock-

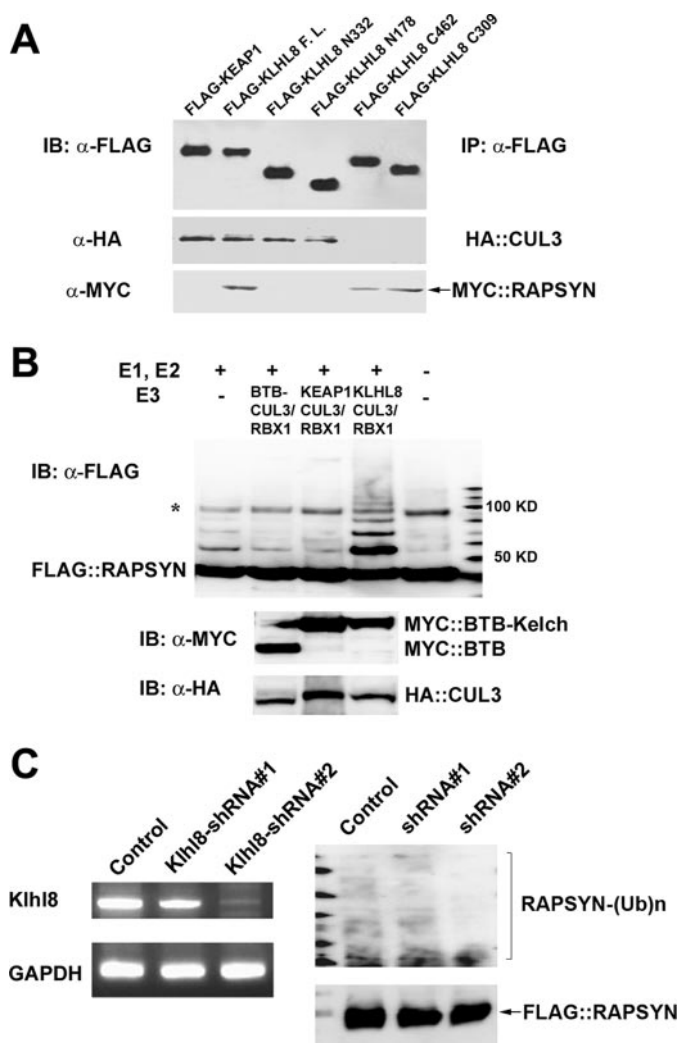


FIGURE 5. The *in vitro* ubiquitination of mammalian rapsyn by CUL3-containing E3 ligase and the effect of KLHL8 knockdown on the ubiquitination of rapsyn. A, the physical interaction between KLHL8, CUL3, and rapsyn. 293T cells transiently expressing epitope-tagged KEAP1, KLHL8 (*KLHL8 F.L.*), the BTB domain of KLHL8 (*KLHL8 N332* (1–332 amino acids), and *KLHL8 N178* (1–178 amino acids)), and the Kelch domain of KLHL8 (*KLHL8 C462* (159–620 amino acids) or *KLHL8 C309* (312–620 amino acids)) were used. The BTB domain alone can interact with CUL3 (lanes 3 and 4, middle panel); the Kelch domain alone can interact with rapsyn (lanes 5 and 6, bottom panel). B, the *in vitro* ubiquitination of rapsyn by CUL3-containing E3 ligase. Purified recombinant human rapsyn was incubated with immunoprecipitated E3 ligase complexes containing the BTB domain of KLHL8 alone (lane 2), full-length KEAP1 (lane 3), or full-length KLHL8 (lane 4) in the presence of purified E1, E2, and Ub proteins. The reaction mixture in lane 1 did not contain the E3 ligase complex, and lane 5 contained the purified rapsyn protein alone. The asterisk indicates a nonspecific product. C, the knockdown of KLHL8 inhibited the ubiquitination of rapsyn in 3T3 cells. IB, immunoblot; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

down animals in an otherwise wild-type background. Interestingly, these animals exhibited phenotypes similar to those of *rpm-1* or *unc-29* mutants, as they showed resistance to the AChR agonist levamisole as manifested by muscle hypercontraction and acceleration of egg laying (Fig. 6, supplemental Table S2). These results indicate that accumulation of excess RPY-1 interferes with either the proper functioning of rapsyn-receptor complexes or their transport to the plasma membrane. Finally, we examined whether transgenic RPY-1 overexpression in WT animals confers resistance to levamisole by estab-

Stability Control of Rapsyn

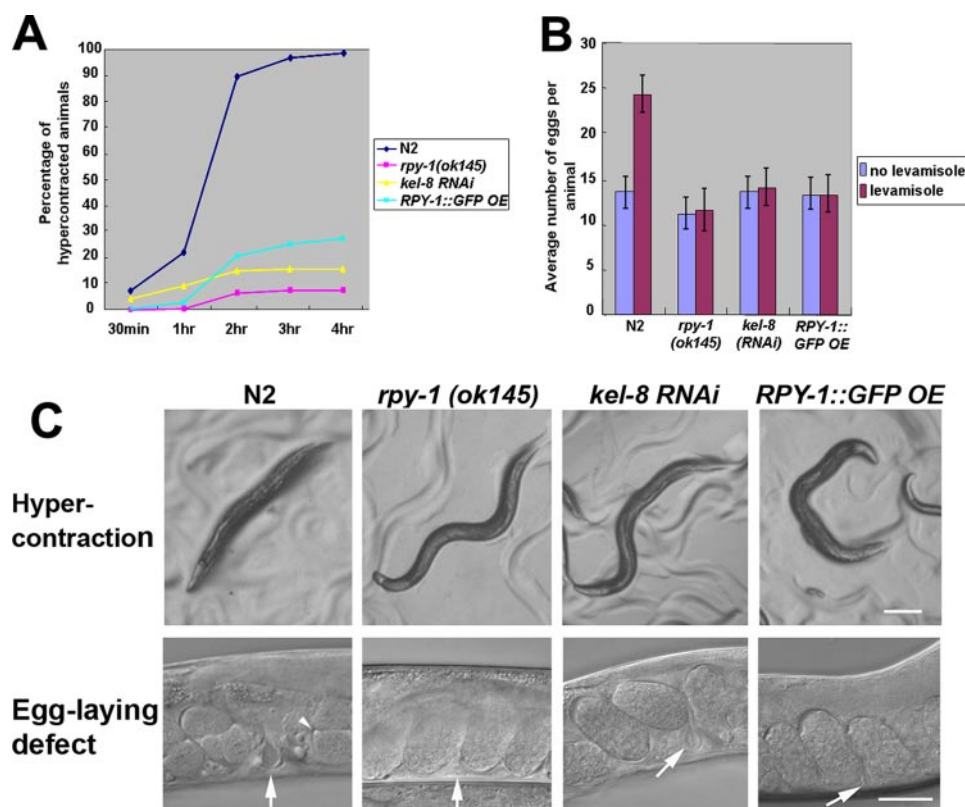


FIGURE 6. Suppression of *kel-8* and overexpression of RPY-1 cause phenotypes similar to that of the *rpy-1* mutation. *A* and *B*, muscle hypercontraction (*A*) and enhanced egg laying (*B*) in the *kel-8 RNAi* and RPY-1::GFP-overexpressing animals. N2 and *rpy-1(ok145)* animals were used as controls. *C* shows representative images of the phenotypes from *B* and *C*. The upper panels show muscle hypercontraction, and the bottom panels show enhanced egg laying after levamisole treatment. The arrows indicate the locations of the vulvae. The arrowhead indicates a two-cell stage egg. The scale bars represent 100 (top row) and 25 μm (bottom row).

lishing a transgenic line injected with the RPY-1::GFP reporter at a concentration of 200 $\mu\text{g}/\text{ml}$, which was an 8-fold higher concentration than that used in the previous experiment. Quantitative PCR experiments showed that different concentrations of transgenes gave rise to different gene doses of the transgenes (supplemental Fig. S8). We observed levamisole resistance in animals injected with the RPY-1::GFP reporter at a concentration of 200 $\mu\text{g}/\text{ml}$ (Fig. 6, supplemental Table S2).

DISCUSSION

In this study, we elucidated the mechanism by which the abundance of rapsyn protein in muscles is regulated. We first proved that *rpy-1*, a putative nematode rapsyn homolog, is a functional homolog of rapsyn. It is interesting to note that agrin and MUSK, two key regulators of receptor clustering in mammals, are apparently not conserved in the nematode (37, 38). A nematode-specific protein such as LEV-10 is required for nAChR clustering in *C. elegans*, indicating that nonconserved proteins may play roles that are similar to those of the agrin-MUSK pathway in mammals (63). It is thus conceivable that rapsyn is the most ancient partner of AChRs. We also showed that RPY-1 stability is dependent on the presence of a non- α subunit of the receptor UNC-29, without which RPY-1 is degraded by the ubiquitin-proteasome pathway. We showed that the CUL-3-containing E3 ligase and KEL-8 are required for degradation of RPY-1. We also showed that RPY-1 degradation

occurs under physiological conditions; failure of this degradation may cause phenotypes similar to those caused by a loss-of-function mutation of *rpy-1*. The evolutionary conservation of the mechanism of rapsyn abundance control suggests that the failure to regulate rapsyn abundance in muscles may cause symptoms of myasthenic syndromes in humans.

Our results show that most RPY-1 proteins in muscle cells were significantly destabilized in the absence of UNC-29 but not in the absence of other nAChR subunits. This finding raises the possibility that the UNC-29-like non- α subunit is specifically involved in functional interactions with RAPSYN for the maintenance of stability in mammals. Furthermore, a specific *unc-29* mutant allele, *e193*, but not other alleles, destabilized RPY-1. Although it is still not clear whether UNC-29 directly interacts with RPY-1, the allele specificity of UNC-29 for RPY-1 degradation provides a genetic argument for a physical interaction between UNC-29 and RPY-1. It is possible that the *unc-29(e193)* mutation

caused a conformational change in UNC-29 or that the Pro to Ser mutation prevented proper membrane positioning of the receptors, thereby preventing the interaction with RPY-1. Mutations in the extracellular domain or in the M4 domain did not affect RPY-1 stability, suggesting that, although they caused a functional defect in the receptors, these mutations did not affect the interaction with RPY-1. Further examination of the regions within UNC-29 and the other nAChR subunits revealed that the cytoplasmic loop of UNC-29 was required for the stable maintenance of RPY-1.

We found most players involved in rapsyn abundance control (CUL-3, RBX-1, KEL-8, Nedd8, and UBC-12) to be components of E3 ligase. Although recent studies have suggested that RING domains often act as E3-Ub ligases for a variety of proteins (64–69), it is unlikely that rapsyn alone acts as the major E3 ligase for its own ubiquitination, because suppression of CUL-3 and other factors in the complex resulted in rapsyn stabilization, even in the presence of the intact RING domain. A similar case was reported for the polycomb protein Ring1B, where self-ubiquitination was not required for Ring1B degradation; degradation occurred through exogenous E3 ligases (70). It is possible that the RING domain of RPY-1 can be protected from KEL-8-mediated ubiquitination if it is masked by physical association with the receptors.

Schaefer and Rongo (71) reported that KEL-8 is involved in the degradation of glutamate receptors in neurons. However,

KEL-8 did not directly interact with the glutamate receptors as the substrate adaptor. The direct target of KEL-8 was not identified in that study. From our results, it is conceivable that KEL-8 interacts with a yet-to-be identified protein that is associated with glutamate receptors. This hypothesis could be tested by identifying proteins that physically interact with KEL-8. In contrast to rapsyn (RPY-1), which shows tissue-specific expression, KLHL8 (KEL-8) is expressed in most tissues of mammals and *C. elegans*, implying that rapsyn is not the only substrate of the KLHL8/CUL3 complex (data not shown). It is also interesting that neuronal RPY-1 was not degraded in the *unc-29* (*e193*) mutant animals. UNC-29 is a muscle-specific nAChR subunit, and the nematode contains many nAChR subunits whose exact expression patterns are not completely known. It is possible that RPY-1 interacts with, and plays a role in clustering, other nAChR subunits in neurons.

Our data show that different concentrations of the *rapy-1* transgene caused different effects; the transgene was able to rescue the *rapy-1* mutant phenotype at a moderate concentration, but it mimicked the *rapy-1* mutation at a higher concentration. Our interpretation is that highly excessive amounts of rapsyn inhibited receptor clustering. Although the presence of rapsyn at the postsynaptic membrane is absolutely required for AChR clustering *in vivo* (12, 18, 19, 72), there have been conflicting reports about the effect of rapsyn abundance on AChR clustering and function. In some studies, expression of exogenous rapsyn stabilized AChR clustering, and in other cases, excessive expression of rapsyn inhibited the clustering of receptors (28, 29, 73). The latter cases are consistent with our results. Because the turnover rate of rapsyn is faster than that of AChR (74, 75), it is conceivable that moderate overexpression of rapsyn might increase the opportunity for rapsyn proteins to interact with the receptors, thus stabilizing more receptors. However, if rapsyn expression is too high, self-aggregation may inhibit clustering of the receptors by interfering with endogenous clustering. Collectively, these data strongly suggest that rapsyn abundance must be tightly regulated for proper muscle function.

It is known that multiple pathways of ubiquitin conjugation are activated in atrophying muscle (76). For example, the muscle-specific ubiquitin ligases MAFbx and MuRF1 are overexpressed during muscular atrophy (77). These E3 ligases are responsible for degradation of structural proteins such as titin and transcription factors such as MyoD within muscles (78). Our finding that CUL-3-containing E3 ligase is involved in the degradation of rapsyn proteins within muscles raises the possibility that deregulation of the CUL-3 ligase complex in muscles could be associated with muscular disorders. Our data suggest that any defects leading to rapsyn depletion or excessive rapsyn accumulation could cause muscle defects similar to those seen in CMS in humans. CMS is caused by genetic defects, such as mutations in AChR, acetylcholinesterase, choline acetyltransferase, the muscle sodium channel SCN4A, MuSK, and rapsyn genes; however, many patients do not have defects in any of these genes (25, 79–82). It is thus possible that factors that affect the abundance of rapsyn could be causal factors in CMS. In this respect, it might be worthwhile to examine these

patients for a new causal relationship between the CUL-3-E3 ligase complex and congenital myasthenic syndromes.

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