Received: 6 January 2021 | Revised: 19 November 2021 | Accepted: 23 November 2021

DOI: 10.1096/fj.202100031RR

THE FASEB JOURNAL

RESEARCH ARTICLE

Ubiquitin ligase TRIM32 promotes dendrite arborization by mediating degradation of the epigenetic factor CDYL

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Funding information
National Natural Science Foundation of China (NSFC), Grant/Award Number: 31530028, 31720103908, 32030052 and 81821092; Ministry of Science and Technology of the People's Republic of China (MOST), Grant/Award Number: 2017YFA0701302

Abstract

Proper dendritic morphology is fundamental to nerve signal transmission; thus, revealing the mechanism by which dendrite arborization is regulated is of great significance. Our previous studies have found that the epigenetic molecule chromodomain Y-like (CDYL) negatively regulates dendritic branching. Current research mostly focuses on the processes downstream of CDYL, whereas the upstream regulatory process has not been investigated to date. In this study, we identified an upstream regulator of CDYL, the E3 ubiquitin ligase tripartite motif-containing protein 32 (TRIM32), which promotes dendrite arborization by mediating the ubiquitylation and degradation of CDYL. By using mass spectrometry and biochemistry strategies, we proved that TRIM32 interacted with CDYL and mediated CDYL ubiquitylation modification in vivo and in vitro. Overexpressing TRIM32 decreased the protein level of CDYL, leading to an increase in the dendritic complexity of primary cultured rat neurons. In contrast, knocking down TRIM32 increased the protein level of CDYL and decreased the dendritic complexity. The truncated form of TRIM32 without E3 ligase activity (ΔRING) lost its ability to regulate dendritic complexity. Most importantly, knockdown of CDYL abolished the reduced complexity of dendrites caused by TRIM32 knockdown, indicating that the TRIM32-mediated regulation of dendritic development depends on its regulation of downstream CDYL. Hence, our findings reveal that TRIM32 could promote dendrite arborization by mediating CDYL degradation. This work initially defines a novel biological role of TRIM32 in regulating mechanisms upstream of CDYL and further presents a potential therapeutic target for the treatment of CDYL-related neurodevelopmental disorders.

KEYWORDS
CDYL, degradation, dendrite arborization, TRIM32, ubiquitylation

Abbreviations: BBS, Bardet-Biedl syndrome; BDNF, brain derived neurotrophic factor; cDNA, complementary DNA; CDS, coding sequence; CDYL, chromodomain Y-like; Co-IP, co-immunoprecipitation; DIV, day in vitro; DMEM, Dulbecco's Modified Eagle Medium; EZH2, enhancer of zeste homolog; FBS, fetal bovine serum; IB, immunoblotting; IP, immunoprecipitation; LGMD2H, limb girdle muscular dystrophy 2H; RA, retinoic acid; RohA, ras homolog gene family, member A; SD, Sprague-Dawley; SETDB1, SET domain bifurcated 1; shRNA, short hairpin RNA; TRIM32, tripartite motif-containing protein 32; Ub, ubiquitin; WT, wild-type.

Lei Liu and Ting-Ting Liu contributed equally to this work.

1 | INTRODUCTION

In the developing brain, dendrites undergo dynamic changes in morphology, forming synapses and neural circuits, which are the basis for the correct functioning of the brain. Given the importance of proper dendritic structures for neuronal functions, abnormal dendritic branches can lead to reduced or excessive synaptic connections, impaired cognitive function, and decreased memory acquisition and motor coordination, and abnormal dendritic branches are associated with neurodevelopmental conditions, including autism and schizophrenia, and neurodegenerative diseases, such as Alzheimer's disease.[1–4] Understanding the molecular mechanism of the regulation of dendritic development will help reveal how circuit functional properties arise during development and explain the causes of some neurodevelopmental diseases.

The epigenetic molecule chromodomain Y-like (CDYL) plays a vital role in the process of dendrite arborization. CDYL was cloned in 1999 and is involved in mouse spermatogenesis.[5] Subsequent studies found that CDYL is an epigenetic factor with chromatin binding ability,[6–8] which usually functions as a transcriptional corepressor.[9–11] CDYL can recruit histone methyltransferases G9a (H3K9me1/2), SETDB1 (H3K9me3) or EZH2 (H3K27me3) to methylate chromatin and inhibit downstream gene transcription.[7,11] A recent report confirmed that CDYL also has crotonase activity.[12,13] According to reports, CDYL is involved in many biological processes, such as sperm formation, X chromosome inactivation, and tumor cell transformation inhibition.[8,11,13] In addition to the above functions, our previous results showed that the epigenetic factor CDYL is also involved in the regulation of neurodevelopment. CDYL can negatively regulate the development of dendrite branches by recruiting EZH2 and inhibiting the expression of downstream brain-derived neurotrophic factor (BDNF).[14] In addition, CDYL regulates neuronal migration by repressing the transcription of the ras homolog gene family member A (RhoA).[15] Another study found that CDYL plays an important role in regulating intrinsic neuroplasticity through repression of axonal Nav1.6 sodium channel expression.[16] Although it has been clearly shown that CDYL acts as a transcriptional corepressor to inhibit downstream target gene expression, the upstream regulators of CDYL are still unclear.

Here, we reported that tripartite motif-containing protein 32 (TRIM32), which has E3 ligase activity, is critically involved in dendritic arborization by regulating CDYL. TRIM32 belongs to a member of the TRIM-NHL protein family. The N-terminus of TRIM32 contains a RING finger domain, two B-boxes, and a coiled-coil domain, while the C-terminus contains NHL repeats.[17] These different protein domains potentially endow TRIM32 protein functional flexibility. TRIM32 functions as a ubiquitin ligase through the RING domain or regulates the activity or stability of the target protein through the NHL domain.[18,19] TRIM32 negatively regulates p53 and c-Myc through ubiquitylation modification to promote the tumorigenesis and differentiation of neural stem cells.[19,20] Moreover, in the case of retinoic acid (RA), TRIM32 interacts with the retinoic acid receptor RARα and enhances its protein stability and transcriptional activity, thereby promoting neuronal differentiation.[18] In addition to its role in neurodevelopment and tumorigenesis, TRIM32 plays an important regulatory role in myogenic cell proliferation and differentiation and muscle regeneration.[21,22] Furthermore, the abnormal expression of TRIM32 is also closely related to many human diseases. The point mutation TRIM32 (P130S) in the B-box domain is associated with Bardet-Biedl syndrome (BBS),[23] and multiple types of mutations in the NHL domain, such as TRIM32 (D487N), (R394H), (D588del), (T520TfsX13), and (R136Stop), were identified in limb girdle muscular dystrophy 2H (LGMD2H) patients.[24–26] In addition, epidermal lesion tissue cells of patients with psoriasis contain abnormally high expression of TRIM32,[27] and high TRIM32 mRNA expression levels were detected in the occipital region of patients with Alzheimer's disease.[28] Although it is clear that TRIM32 has the function of ubiquitin ligase, its downstream target protein has rarely been identified, and the biological function of this protein remains to be determined.

In this study, we showed that CDYL interacts with TRIM32, which was confirmed in vivo and in vitro. Moreover, TRIM32 can mediate CDYL ubiquitylation modification in vivo and in vitro, which relies on the RING finger domain of TRIM32. TRIM32 promotes CDYL degradation by ubiquitylation. Furthermore, TRIM32 positively regulates dendritic branching by negatively regulating the level of CDYL. Together, our findings reveal an upstream regulatory protein of CDYL, TRIM32, which is critical for dendritic arborization.

2 | MATERIALS AND METHODS

2.1 | Animals

Male Sprague-Dawley (SD) rats weighing 250 g, female SD rats at 18 days of pregnancy and female ICR mice at 14.5 days of pregnancy were purchased from the Animal Center of Peking University Health Science Center. The rats were raised in climate-controlled rooms (temperature: 24 ± 2°C and humidity: 50 ± 5%) on a 12-h light-dark cycle (light on 8:00 a.m.) with free access to food and water.

2.2 | Plasmids

Flag-CDYL and CDYL shRNAs have been previously described.[14] The rat CDYL coding sequence (CDS) was amplified by PCR from the Flag-CDYL plasmid and subcloned into the pGEX-5X-1 vector, namely, GST-CDYL. The rat TRIM32 CDS and its deletion mutants were amplified by PCR from rat cDNA and subcloned into the pET-28a (+) vectors or pcDNA™3.1/myc-His(−) A vectors, namely, His-TRIM32, His-ΔRING, His-ΔNHL, His-NHL, Myc-TRIM32 and Myc-TRIM32 (ΔRING). pXJ40-HA-Ub was a gift from the laboratory of Dr. Li at Peking University. The relevant primers for constructing the above vectors are listed in Table S1. Sequences for siRNA oligos against TRIM32 were as follows: ShRNA1: 5′-CCGGGCCACTTCTTCTCGGAGAATGCTCGAGCATTCTCCGAGAAGAAGTGGCTTTTTG-3′ and 5′-AATTCAAAAAGCCACTTCTTCTCGGAGAATGCTCGAGCATTCTCCGAGAAGAAGTGGC-3′; ShRNA2: 5′-CCGGATAACTCCCTCAAGGTATATACTCGAGTATATACCTTGAGGGAGTTATTTTTTG-3′ and 5′-AATTCAAAAAATAACTCCCTCAAGGTATATACTCGAGTATATACCTTGAGGGAGTTAT-3′; ShRNA3: 5′-CCGGTGAAGTTGAGAAGTCCAATAGCTCGAGCTATTGGACTTCTCAACTTCATTTTTG-3′; and 5′-AATTCAAAAATGAAGTTGAGAAGTCCAATAGCTCGAGCTATTGGACTTCTCAACTTCA-3′. The shRNA1, 2, and 3 vectors against TRIM32 were constructed by inserting the above sequences for TRIM32 siRNA into the pLKO.1 lentiviral shRNA vector. To create TRIM32 shRNA3-resistant TRIM32 (TRIM32-R), the third nucleotide of each codon in the target sequence was mutated without changing the identity of the amino acids.

2.3 | Cell culture and transfection

HEK293T cells were cultured in DMEM (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin solution (100×). When the cell density reached 60%–70%, cells were transfected with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions.

2.4 | IP assays and western blotting

For coimmunoprecipitation of endogenous TRIM32 and CDYL, rat cerebral cortex lysates were incubated with anti-CDYL or anti-TRIM32 antibodies followed by incubation with Protein A/G agarose beads. The immunoprecipitates were subjected to western blot assays with anti-TRIM32 or anti-CDYL antibodies.

Standard western blot assays were performed to analyze protein expression. The protein concentration of each sample was determined using BCA protein assay reagent (Pierce, Rockford, IL, USA) and BSA as a standard. Equal amounts of samples were boiled for 10 min with SDS-PAGE sample buffer, separated using 10% SDS-PAGE, and then transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk in TBST for 1 h at room temperature and then incubated overnight at 4°C with primary antibody. The next day, the blots were washed 5× (5 min/wash) in TBST. The membranes were then incubated with horseradish–conjugated secondary antibody for 1 h at room temperature. Finally, the blots were developed with a chemiluminescence kit (WBKLS0500, Millipore, USA).

The primary and secondary antibodies used in this study were as follows: CDYL (1:2000, HPA035578, Sigma, USA), TRIM32 (1:3000, 10326-1-AP, Proteintech, USA), Flag (1:2000, F1804, Sigma, USA), Myc (1:3000, 60003-2-Ig, Proteintech, USA), GST (1:1000, C1303; Applygen Technologies Inc., China), His (1:1000, C1301; Applygen Technologies Inc., China), HA (1:3000, sc-805, Santa Cruz Biotechnology, USA), β-actin (1:2000, TA-09, Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd, China) and HRP-conjugated secondary antibody (1:5000, goat anti-rabbit, rabbit anti-goat, or goat anti-mouse; Bio-Rad Laboratories, Hercules, CA, USA).

2.5 | In vitro pull-down assays

For recombinant protein expression, TRIM32 and its deletion mutants were introduced into pET28a-His, and CDYL was introduced into pGEX-5x-1-GST. His-tagged TRIM32 or its mutants were coexpressed with GST or GST-CDYL in *Escherichia coli* BL21 (DE3). Protein expression was induced at 16°C with 0.3 mM isopropyl β-D-1-thiogalactopyranoside overnight. GST or GST-CDYL was purified by glutathione sepharose according to the manufacturer's protocol (GE Healthcare Life Sciences, Buckinghamshire, UK) and detected by western blotting. His-tagged recombinant proteins were purified by Ni-NTA according to the manufacturer's protocol (Cat# 30 210, Qiagen. Valencia, CA, USA). The resulting proteins were separated using SDS-PAGE and detected by western blotting.

2.6 | In vitro ubiquitylation assays

To assess the ubiquitylation of CDYL by TRIM32 in vitro, we used an ubiquitinylation kit (BML-UW9920, Enzo, Lausen, Switzerland). According to the manufacturer's

protocol, 2.5 μM Ub, 100 nM E1, 2 μM E2, 5 mM Mg-ATP, 100 nM His-TRIM32, and 1 μM GST-CDY were added to every reaction system and then incubated at 37°C for 30–60 min. Finally, the reaction results were analyzed by western blotting. Horseradish peroxidase-conjugated streptavidin (HRP-SA, 1:3000, SA00001-0, Proteintech, USA) was used to detect ubiquitylation of CDYL.

2.7 | In vivo ubiquitylation assays

These assays were performed using the HEK293T cell line. HEK293T cells were transfected with indicated plasmids expressing Flag-CDYL (1 μg), Myc-TRIM32 (6 μg) or Myc-TRIM32 (ΔRING) (6 μg) and HA-tagged Ub (pXJ40-HA-Ub, 1 μg) either alone or in combination with Lipofectamine 2000. Twenty-four hours after transfection, cells were treated with MG132 (20 μM, Selleckchem, Houston, TX, USA) for 12 h to block 26S proteasome-mediated degradation. Then, the cells were lysed using lysis buffer. After the cells were fully lysed, the lysate was centrifuged to take the supernatant for the next step. To analyze the ubiquitylation of CDYL, CDYL in supernatant was immunoprecipitated by anti-Flag-M2 agrose and evaluated by western blot analyses. CDYL protein and ubiquitinated CDYL bands were detected by anti-Flag antibody and anti-HA antibody, respectively.

2.8 | Culture and transfection of primary neurons

The SD rats were decapitated at 18 days of pregnancy, and the fetuses were quickly removed. The intact brain tissue of the embryo was quickly removed. The hippocampus was isolated under a microscope and digested with 0.2% trypsin at 37°C for 30 min. The dissociated neurons were uniformly seeded on a 35 mm culture dish coated with poly-D-lysine (Sigma, USA) and containing DMEM with 10% FBS at a density of 7×10^5 cells per dish. After 4 h of incubation at 37°C, the medium was replaced with neurobasal medium (GIBCO, Grand Island, NY, USA) supplemented with 2% B27 (Invitrogen, Carlsbad, CA, USA) and 0.5 mM GlutaMAX-1 (GIBCO, Grand Island, NY, USA). After that, half of the medium was changed every three days. To inhibit the growth of glia, 10 μM cytarabine was added to the culture medium when changing the medium on the third day.

When the neurons were cultured to the eighth day in vitro (DIV), transfection experiments were performed. Neurobasal medium was preheated at 37°C for four hours before transfection. Lipofectamine 2000 was used for neuronal transfection according to the manufacturer's protocol. Each petri dish was transfected with 2 μg of plasmids. The ratio of plasmids:Lipofectamine 2000 = 1:1.5, and 3 μl of Lipofectamine 2000 was needed for each petri dish. Three days after transfection, morphological analysis was performed.

2.9 | In utero electroporation

Female ICR mice at embryonic 14.5 days were used to perform electroporation. Mice were anesthetized by intraperitoneal injection with 0.7% sodium pentobarbital and checked for vaginal plugs. Mice embryos were exposed in the uterus and 1 μl of DNA solution was injected into the lateral ventricle through the uterus wall, followed by electroporation. Plasmids were prepared in Milli-Q water at a concentration of 1 μg/μl for pEGFP-N1, 3 μg/μl for shRNAs, and 6 μg/μl for Flag-CDYL, Myc-TRIM32, Myc-TRIM32 (ΔRING) or TRIM32-R. Electrical pulses of 36 V were generated with an ElectroSquireportator T830 (BTX) and applied to the cerebral wall for 50 ms each, for a total of five pulses, at intervals of 950 ms. The uterus was then replaced, and the abdomen wall and skin were sutured. After surgical manipulation, mice were allowed to recover to consciousness in a 37°C incubator.

2.10 | Analysis of neuronal morphology

pEGFP-N1 plasmids were cotransfected to clearly show the morphology of neurons, and neurons were selected by GFP expression. Selected neurons were photographed at 20× magnification using an inverted Leica confocal microscope (Leica TCS STED scanner, Germany). To analyze the length of the total dendritic branches, all arbors and their branches were traced, and their length was measured using ImageJ software. For convenience, the control group was set to 100%, and the experimental group was normalized to the control group. For Sholl analysis, concentric circles were drawn with a diameter difference of 15 μm around the neuron cell body, and the number of dendrites passing through each circle was manually counted.[29,30]

2.11 | Statistical analysis

Statistical analyses were performed with the Prism 7.0 software (GraphPad, La Jolla, CA, USA). All data are represented as the mean ± SEM. Comparisons between two groups were made using Student's *t* tests. Comparisons among three or more groups were made using one-way ANOVA followed by Bonferroni's multiple comparisons

test. A threshold of $p < .05$ indicated statistical significance. Data marked with asterisks in the figures are significantly different from the control as follows: $*p < .05$, $**p < .01$, and $***p < .001$.

3 | RESULTS

3.1 | CDYL interacts with TRIM32

To gain insights into the biological function and identify the upstream regulatory molecules of CDYL, we carried out affinity purification of CDYL from cerebral cortex lysates using an anti-CDYL antibody to isolate CDYL-associated proteins. Mass spectrometry analysis identified more than 20 possible associated proteins (Table S2). One of the candidate proteins, TRIM32, is an E3 ubiquitin ligase that regulates target proteins through ubiquitylation. Therefore, we speculated that TRIM32 may be an upstream regulatory protein of CDYL.

To test this hypothesis, we first validated the interaction between TRIM32 and CDYL. TRIM32 can be immunoprecipitated by CDYL from the rat brain, and vice versa, indicating that TRIM32 and CDYL are present in a robust complex (Figure 1A). Moreover, the direct interaction between TRIM32 and CDYL was confirmed by GST and Ni-NTA pull-down assays in vitro (Figure 1C,D). TRIM32 contains several key domains, including a RING finger, which confers E3 ubiquitin ligase activity, two B-box domains and a coiled-coil motif, which mediate oligomerization, and a C-terminal NHL domain, which was reported to be required for the interactions of TRIM32 with other proteins (Figure 1B).[20,31,32] To confirm the binding domain of TRIM32 with CDYL, several His-tagged deletion mutants of TRIM32 were constructed, including ΔRING, ΔNHL, and NHL (Figure 1B). The pull-down assays showed that the WT, ΔRING, ΔNHL, and NHL mutants of TRIM32 all interacted with CDYL (Figure 1C,D). These results proved that in addition to the NHL domain binding to CDYL, the N-terminus of TRIM32 also binds to CDYL. These results together demonstrated that TRIM32 interacts with CDYL in the brain, and both the N-terminus and C-terminus of TRIM32 can directly interact with CDYL.

3.2 | TRIM32 promotes ubiquitylation of CDYL

TRIM32 has E3 ubiquitin ligase activity and participates in the regulation of some substrates through ubiquitylation modification.[18–20,31–33] Since TRIM32 interacts with CDYL, we further investigated whether TRIM32 mediates the ubiquitylation of CDYL. First, we performed an in vitro ubiquitylation assay. We carried out this assay by using an ubiquitylation kit. Moreover, we purified recombinant TRIM32 and CDYL. In the presence of ATP, Ub, E1 enzyme and the E2 enzyme UbcH2, instead of other E2 enzymes, CDYL can be ubiquitinated by TRIM32 (Figure 2A). The RING finger domain is required for the E3 ubiquitin ligase activity of TRIM32. When the RING finger domain was deleted, the ubiquitylation modification of CDYL by TRIM32 was abolished (Figure 2B).

To investigate whether TRIM32 ubiquitinates CDYL in vivo, HEK293T cells were transfected with Flag-CDYL vectors and HA-Ub vectors together with vectors expressing WT or TRIM32 (ΔRING). These cell lysates were subjected to immunoprecipitation with an antibody against Flag and then analyzed by immunoblotting with an antibody against HA. The results showed that there were obvious smeared bands of ubiquitinated CDYL in the presence of WT TRIM32 but not the TRIM32 (ΔRING) mutant (Figure 2C), suggesting that TRIM32 promotes CDYL ubiquitylation in cells, which depends on the RING finger domain of TRIM32. Taken together, these results indicated that CDYL is a substrate for TRIM32 ubiquitylation activity.

3.3 | TRIM32 promotes CDYL degradation through ubiquitylation

The ubiquitylation of proteins is usually used as a marker of degradation by the 26S proteasome. However, according to current reports, the E3 ubiquitin ligase activity of TRIM32 exerts at least three effects on its substrates, including promoting substrate degradation,[19,20,31,33] stabilizing the substrates,[18] and affecting the binding of the substrates to other proteins.[32] To investigate the specific effects of TRIM32-mediated ubiquitylation on CDYL, HEK293T cells were transfected with TRIM32 shRNA vectors or TRIM32 overexpression vectors. In three independent experiments, cells expressing TRIM32 shRNA1, 2, or 3 all showed a decrease in TRIM32 expression, which ultimately caused an elevation in the levels of CDYL protein (Figure 3A–C). In contrast, overexpressing Myc-TRIM32 in HEK293T cells gave rise to a decrease in CDYL levels (Figure 3D,E). The above results indicated that TRIM32 can promote the degradation of CDYL in cells. To investigate whether TRIM32-mediated degradation of CDYL depends on its E3 ubiquitin ligase activity, HEK293T cells were transfected with wild-type (WT) TRIM32 or TRIM32 (ΔRING) vector. Compared with the promotion

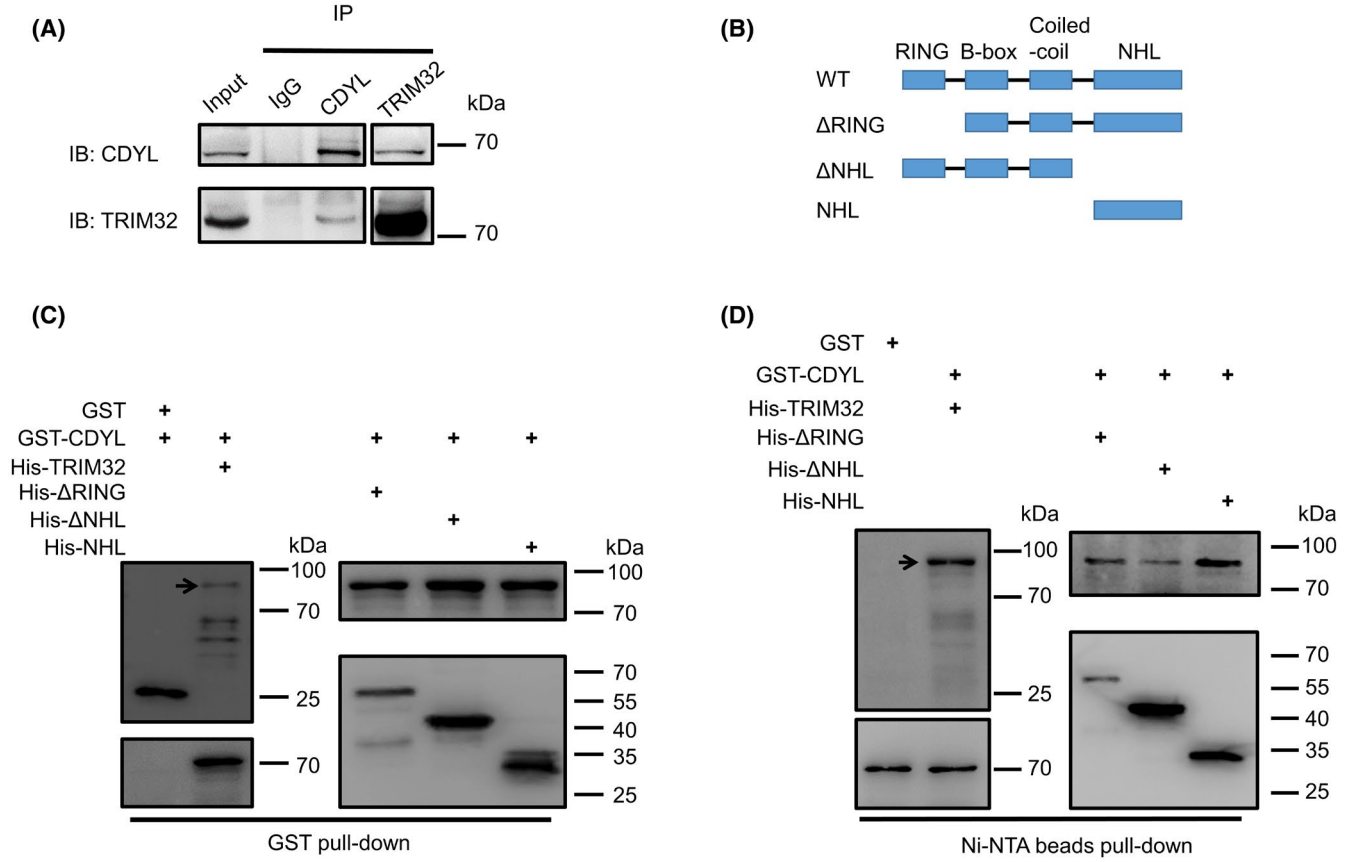

FIGURE 1 Chromodomain Y-like (CDYL) and tripartite motif-containing protein 32 (TRIM32) interact with each other. (A) In vivo immunoprecipitation. CDYL or TRIM32 proteins were purified from rat brain lysates. Purified proteins were immobilized on Protein A/G agarose beads. Bound proteins were detected with anti-TRIM32 or anti-CDYL antibodies. IP, immunoprecipitation; IB, immunoblotting. (**B**) Schematic representation of structural features of WT or serial deletion mutants of TRIM32. (C,D) In vitro pull-down assays. CDYL was fused to the GST tag, and TRIM32 or its deletion mutants were fused to the poly-His tag. His-tagged TRIM32 or its mutants were coexpressed with GST or GST-tagged CDYL in Escherichia coli BL21 (DE3). GST or GST-CDYL was purified from the *E. coli* lysates by GST beads, and the precipitated complexes were examined by western blotting using anti-GST and anti-His antibodies (C); His-TRIM32 or its deletion mutants were purified from the *E. coli* lysates by Ni-NTA beads, and the precipitated complexes were examined by western blotting using anti-GST and anti-His antibodies (D)

of CDYL degradation by WT TRIM32, the overexpression of Myc-TRIM32 (ΔRING) did not cause the same effect (Figure 3F,G). Furthermore, treatment of cells with MG132, a widely used proteasome inhibitor, abrogated the decrease in CDYL protein levels caused by TRIM32 overexpression (Figure 3H,I), suggesting that CDYL degradation caused by TRIM32 occurs through the 26S proteasome pathway.

To investigate whether TRIM32 regulates CDYL levels in neurons, TRIM32 was knocked down by TRIM32 shRNA3 in cultured rat hippocampal neurons (Figure S1A,B). TRIM32 knockdown caused an increase in CDYL (Figure S1A,C). Moreover, Overexpression of TRIM32 led to an increase in CDYL protein level (Figure S1D,E). Similarly, MG132 can inhibit the reduction of CDYL caused by TRIM32 overexpression in neurons (Figure S1F,G). These results confirmed TRIM32-mediated proteasome-dependent degradation of CDYL in neurons. Taken together, the above results demonstrated that TRIM32 can downregulate CDYL through its ubiquitin ligase activity.

3.4 | TRIM32 positively regulates dendritic branching

TRIM32 is involved in some biological processes, such as neuronal differentiation,[18,19,34] tumorigenesis,[20] and muscle regeneration,[21] but it is still unknown whether TRIM32 is involved in dendritic branching. CDYL is known to negatively regulate the development of dendritic branches.[14] Here, we have demonstrated that TRIM32 promotes ubiquitylation and degradation of CDYL. Furthermore, we investigated whether TRIM32 can participate in the branching of neuronal dendrites by culturing, transfecting and analyzing primary

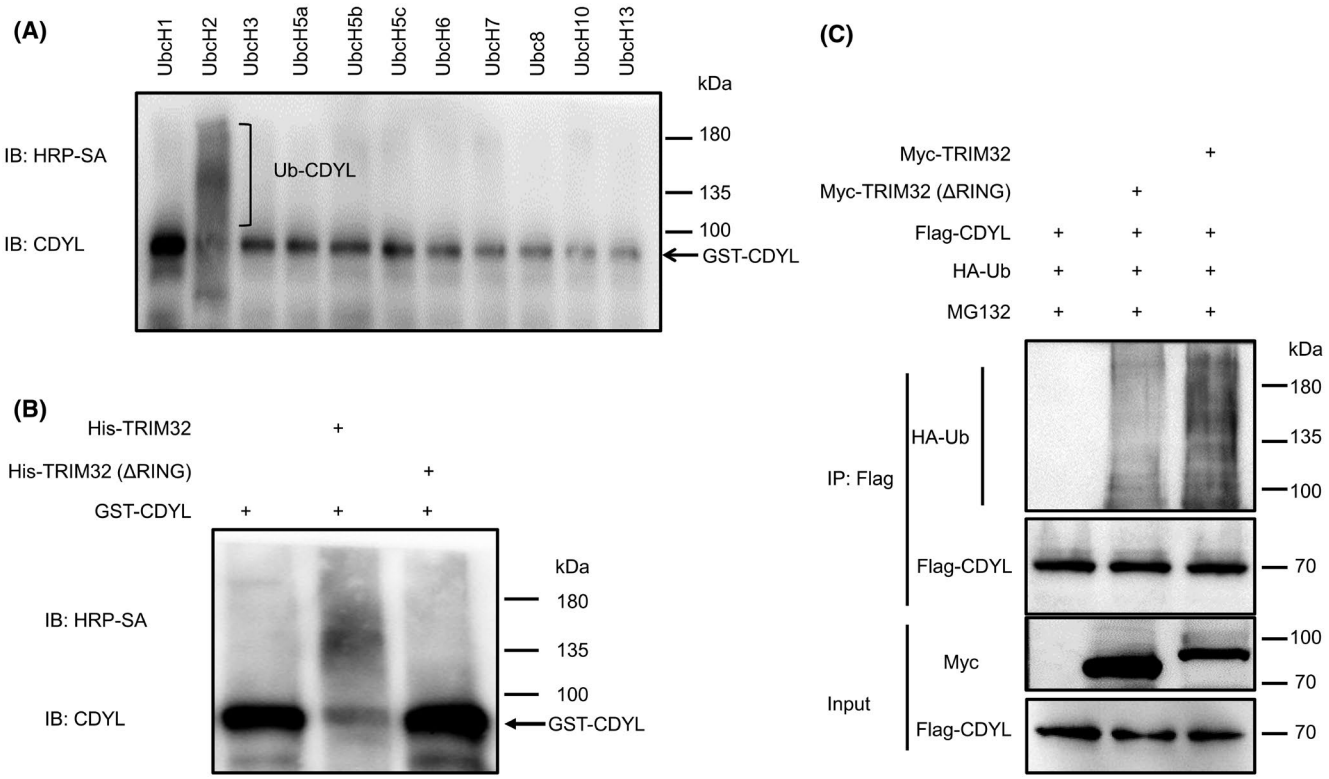

FIGURE 2 Tripartite motif-containing protein 32 (TRIM32) ubiquitinates chromodomain Y-like (CDYL). (A) In vitro ubiquitylation assays of CDYL. Purified recombinant His-TRIM32 and GST-CDYL were incubated in the presence of ATP, ubiquitin, E1 and different E2s as specified in the figure. The resulting proteins were separated using SDS-PAGE and detected by western blotting using anti-GST antibody and HRP-SA. High molecular mass ubiquitin conjugates (ubiquitin-CDYL, Ub-CDYL) accumulated only after incubation of TRIM32 with UbcH2. IB, immunoblotting. (B) The ubiquitylation of CDYL by TRIM32 depends on the RING finger domain of TRIM32 in vitro. In the presence of UbcH2, ATP, ubiquitin, and E1, full-length TRIM32 promoted the formation of ubiquitin conjugates, whereas TRIM32 (ΔRING) did not. IB, immunoblotting. (C) TRIM32, but not the TRIM32 (ΔRING) mutant, promoted ubiquitylation of CDYL in vivo. HEK293T cells were transfected with Myc-TRIM32 or Myc-TRIM32 (ΔRING), Flag-CDYL and HA-Ub. Cell lysates were immunoprecipitated with the Flag antibody and then immunoblotted with the HA antibody

hippocampal neurons. We examined the effect of gain-of-function and loss-of-function TRIM32 on the morphology of neurons. Overexpression of full-length TRIM32 in cultured rat hippocampal neurons led to a significant increase in dendritic branching and total dendritic length compared with the control group (Figure 4A–C). In contrast, knockdown of TRIM32 by shRNA led to a decrease in dendritic branching and total dendritic length (Figure 4A–C). To exclude the possible off-target effects of the shRNA3, rescuing experiments were performed with TRIM32-R, a construct that was derived from WT TRIM32 by silent mutation and therefore resistant to the shRNA3. Expression of TRIM32-R restored the dendritic morphology affected by TRIM32 shRNA, indicating the specific effects of TRIM32 shRNA3 (Figure 4A–C). We also investigated whether the effects of TRIM32 on dendritic branching depend on its Ub ligase activity by transfecting hippocampal neurons with TRIM32 (ΔRING). Unlike that of WT TRIM32, overexpression of TRIM32 lacking the RING domain in cultured hippocampal neurons did not affect dendritic branching (Figure 4A–C). These results indicated that TRIM32 promotes the dendritic complexity of hippocampal neurons, which is dependent on its Ub ligase activity.

Furthermore, we investigated whether TRIM32 has the same role on dendritic branching in mice by in utero electroporation. Specifically, CDYL shRNA, CDYL, TRIM32 shRNA3, TRIM32, TRIM32 (ΔRING), or control together with a GFP construct was transfected to mice progenitor cells at E14.5 via electroporation. Consistent with the in vitro results, TRIM32 promotes dendritic branching, while CDYL has the opposite effect at postnatal day 15 (P15) (Figure 5A–C). Furthermore, rescuing experiments in which TRIM32-R was cotransfected with TRIM32 shRNA3 to mice progenitor cells at E14.5 via electroporation completely restored the normal dendrite morphology of transfected hippocampal neurons at P15 (Figure 5A–C). Overexpression of TRIM32 increased the dendritic complexity of mice neurons, while overexpression of TRIM32 (ΔRING) had no significant effect on dendritic complexity (Figure 5A–C). These data

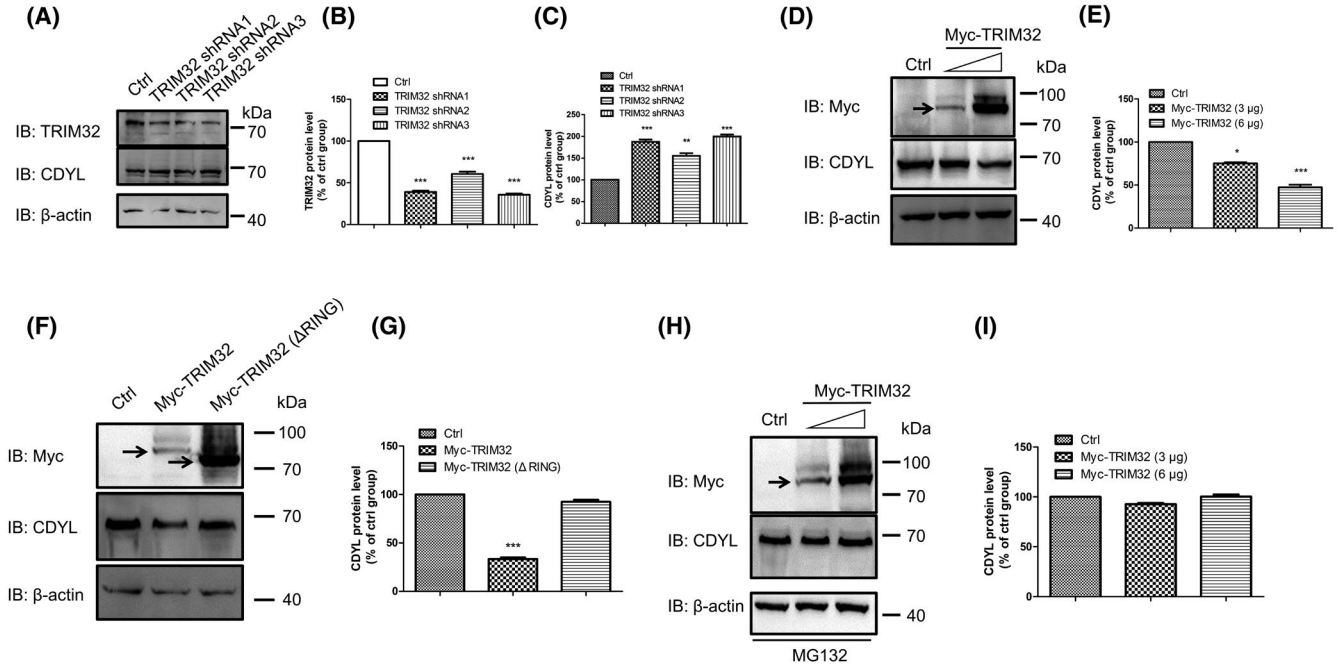

FIGURE 3 Tripartite motif-containing protein 32 (TRIM32) promotes chromodomain Y-like (CDYL) degradation through ubiquitylation. (A) Knockdown of endogenous TRIM32 by shRNA increased the levels of endogenous CDYL protein in HEK293T cells. Three independent TRIM32 shRNAs, TRIM32 shRNA1, TRIM32 shRNA2 and TRIM32 shRNA3, can all cause a reduction in TRIM32 expression, which ultimately leads to an increase in the levels of CDYL protein. (B,C) Quantification of the blots of TRIM32 and CDYL shown in the A ($n = 3$). (D) Ectopic expression of TRIM32 reduced the levels of endogenous CDYL protein in HEK293T cells. HEK293T cells were transfected with Myc-TRIM32 plasmids (3 or 6 μg). Cell lysates were subjected to immunoblot analysis with specific antibodies against CDYL, Myc, and β-actin. (E) Quantification of the blot of CDYL shown in the D ($n = 3$). (F) The degradation of CDYL promoted by TRIM32 was inhibited by MG132. HEK293T cells were transfected with Myc-TRIM32 plasmids (3 or 6 μg). Twenty-four hours after transfection, cells were treated with MG132 (20 μM) for 12 h. Cell lysates were subjected to immunoblot analysis with specific antibodies against CDYL, Myc, and β-actin. (G) Quantification of the blot CDYL shown in the F ($n = 3$). (H) The RING finger domain is responsible for the degradation of TRIM32-mediated CDYL degradation. HEK293T cells were transfected with 3 μg of Myc-TRIM32 or Myc-TRIM32 (ΔRING) plasmids. Cell lysates were subjected to immunoblot analysis with specific antibodies against CDYL, Myc, and β-actin. In all of the above figures, the control group is represented by the control (Ctrl), and IB indicates immunoblotting. (I) Quantification of the blots of TRIM32 and CDYL shown in the H ($n = 3$)

indicated that TRIM32 promotes the dendritic complexity of hippocampal neurons in vivo.

3.5 | TRIM32 promotes dendritic complexity by regulating CDYL

TRIM32 and CDYL have opposite effects on dendritic complexity, and TRIM32 can regulate CDYL protein levels through ubiquitylation. Hence, we further examined whether TRIM32 positively regulates dendritic branching by negatively regulating CDYL. First, we confirmed that CDYL negatively regulates dendritic complexity by analyzing the morphology of hippocampal neurons by transfecting Flag-CDYL or CDYL shRNA vectors (Figure 6A–C). Knockdown of TRIM32 or CDYL can reduce or increase dendritic complexity, respectively. We hypothesized that if CDYL is the key downstream regulator of TRIM32-mediated dendritic branching, then neurons that knock down both CDYL and TRIM32 should exhibit the knockdown phenotype of the downstream molecule, namely, CDYL. To confirm our speculation, we cotransfected TRIM32 and CDYL shRNAs into hippocampal neurons. Unlike the dendritic morphology of cells with TRIM32 knockdown alone, cells with CDYL and TRIM32 knockdown together showed the phenotype of cells with CDYL knockdown alone (Figure 6A–C), indicating that CDYL knockdown abolished the effects of TRIM32 shRNA on dendritic complexity and that CDYL is required for TRIM32-mediated regulation of dendritic branching. Together, our results suggest that TRIM32 promotes dendritic complexity by regulating CDYL expression.

4 | DISCUSSION

TRIM32 is an E3 ubiquitin ligase containing a RING finger domain and is reported to play an important role

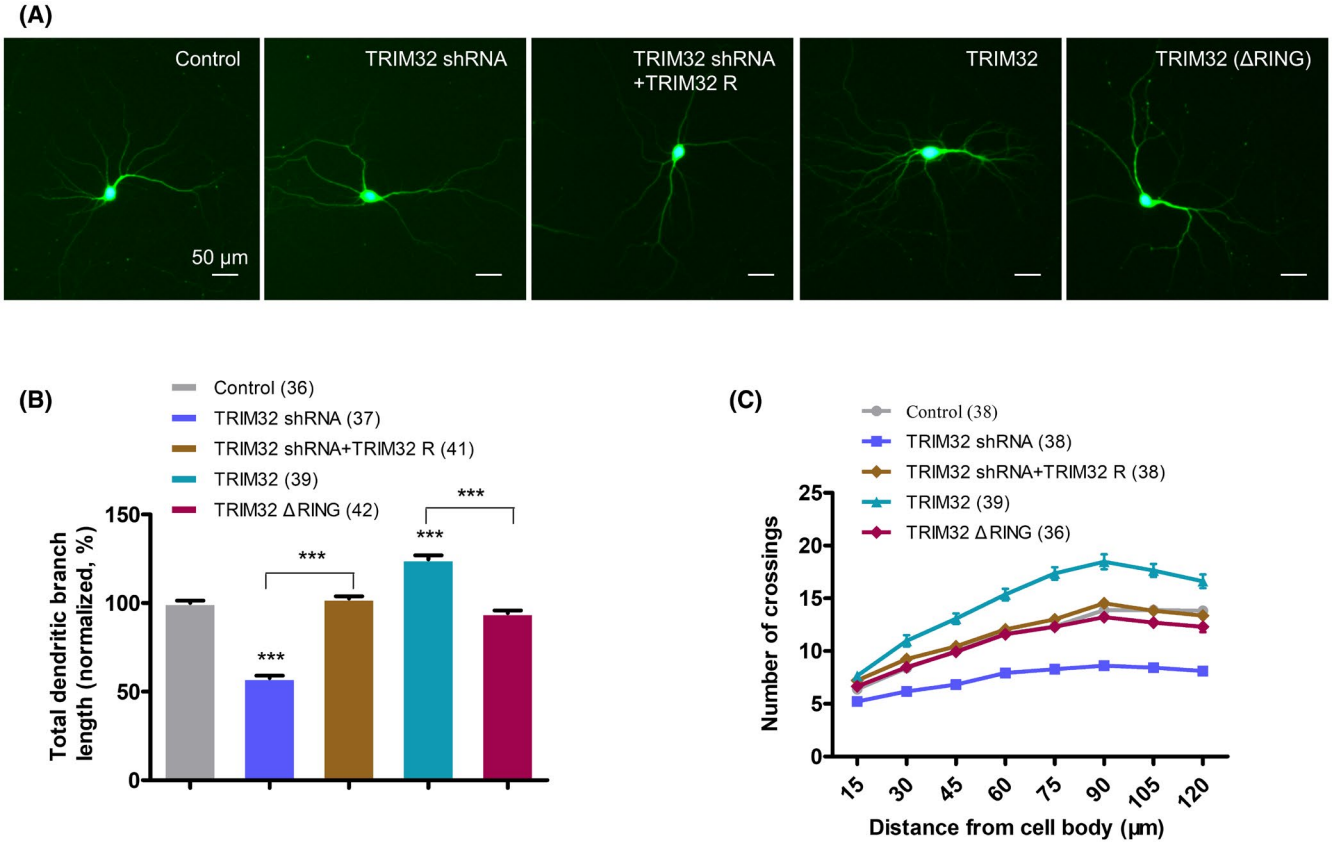

FIGURE 4 Tripartite motif-containing protein 32 (TRIM32) positively regulates dendritic branching. (A) Representative images of cultured hippocampal neurons transfected with control vector, TRIM32 shRNA (TRIM32 shRNA3), TRIM32 shRNA + TRIM32 R, Myc-TRIM32 or Myc-TRIM32 (ΔRING) at DIV 8 for 3 days. Cells were cotransfected with pEGFP-N1 to visualize the morphology of the transfected neurons. Scale bar, 50 μm. (B) Quantification of the total dendritic branch length of hippocampal neurons transfected with the indicated constructs as shown in A. ***$p < .001$, one-way ANOVA with Bonferroni's multiple-comparisons test. The number labeled in the bracket indicates the number of neurons analyzed. Error bars indicate SEM. (C) Sholl analysis of hippocampal neurons transfected with the indicated constructs as shown in A. The number labeled in the bracket indicates the number of neurons analyzed

in the regulation of many different biological processes, such as neural stem cell differentiation, innate immunity, tumorigenesis, myogenic differentiation and muscle regeneration.[18–22,32] In this study, we identified TRIM32 as an upstream regulator of CDYL involved in dendrite complexity. CDYL is known to negatively modulate dendritic complexity by repressing BDNF expression during brain development or neuronal activation,[14] and TRIM32 promotes CDYL degradation through ubiquitylation. Thus, based on these known results, we proposed a TRIM32-CDYL-mediated regulatory pathway for dendritic development (Figure 7). In the process of brain development, CDYL can be ubiquitinated by TRIM32 and then degraded, leading to increased expression of genes related to dendritic branch development and ultimately promoting dendritic branching (Figure 7).

As a transcriptional corepressor, CDYL inhibits downstream gene expression by recruiting histone methyltransferases.[7,9,11,14–16] Several downstream target genes of CDYL have been identified, including BDNF, SCN8A and RhoA, and these genes play a vital role in different biological processes.[14–16] In addition to acting as a transcriptional corepressor, CDYL exerts enoyl hydratase activity to negatively regulate histone crotonylation involved in regulating spermatogenesis.[13] These studies help us understand the functions of CDYL by investigating cooperative molecules, downstream regulatory genes or histone substrates of CDYL. However, before the present study, the upstream regulatory mechanism of CDYL was still unclear. Here, our results showed that CDYL can be ubiquitinated and degraded by an E3 ubiquitin ligase, TRIM32 (Figures 2 and 3). TRIM32 was the first upstream regulator of CDYL to be discovered. Our study deepens our understanding of the molecular function and regulatory mechanism of CDYL. In addition, we know that as the cerebral cortex develops, the level of CDYL gradually decreases,[14] the activation of hippocampal and cortical neurons can lead to the gradual degradation of CDYL, and the degradation process is repressed by MG132 treatment.[14,16] These findings

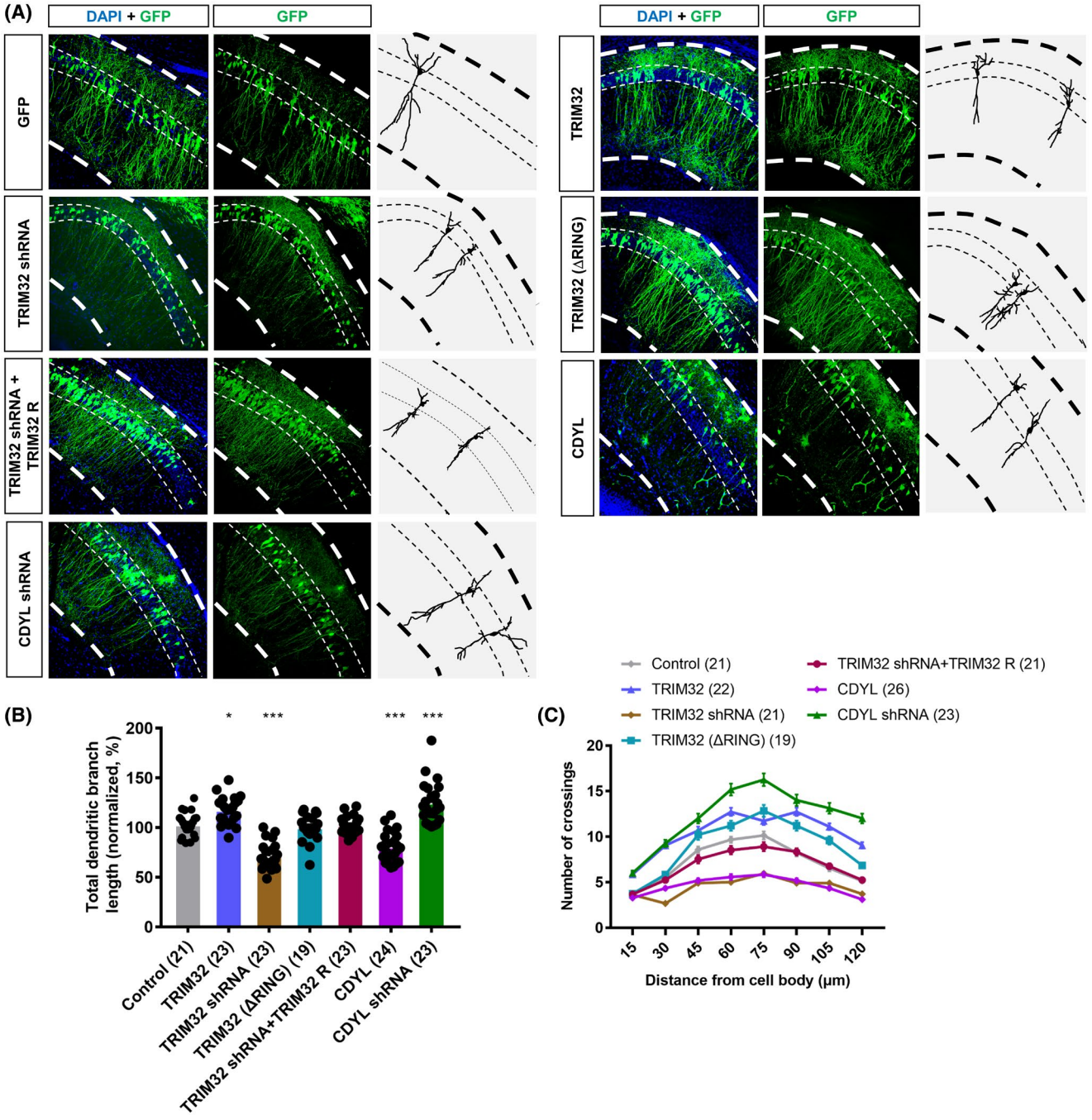

FIGURE 5 Tripartite motif-containing protein 32 (TRIM32) promotes dendritic branching in vivo. (A) Representative images of P15 mice brains, which were transfected with GFP and the indicated constructs by in utero electroporation at E14.5. Transfected cells were photographed at 10× magnification using an inverted Leica confocal microscope. Scale bar, 50 μm. (B) Quantification of the total dendritic branch length of the neurons from hippocampal slices. (C) Sholl analysis of the neurons from hippocampal slices from in utero electroporated brains transfected with the indicated constructs. Analysis was done at a distance of 100 μm from the soma

suggested that there is an E3 ubiquitin ligase upstream of CDYL. Hence, we speculate that TRIM32 may be responsible for this degradation regulation of CDYL.

TRIM32 was first discovered by screening the Tat binding protein of HIV-1 virus and was found to help the Tat protein transcriptionally activate HIV-1 genes.[35] Subsequently, it was found that TRIM32 has E3 ubiquitin ligase activity and participates in some biological processes. At present, only a few substrates of TRIM32 have been identified, including NDRG2, c-Myc, p53, protein kinase C ζ (PKCζ), MITA, and RARα.[18–20,32–34] Some substrates, including NDRG2, c-Myc and p53, are ubiquitinated by TRIM32 and then degraded.[19,20,33] However, TRIM32-mediated ubiquitylation modification does not

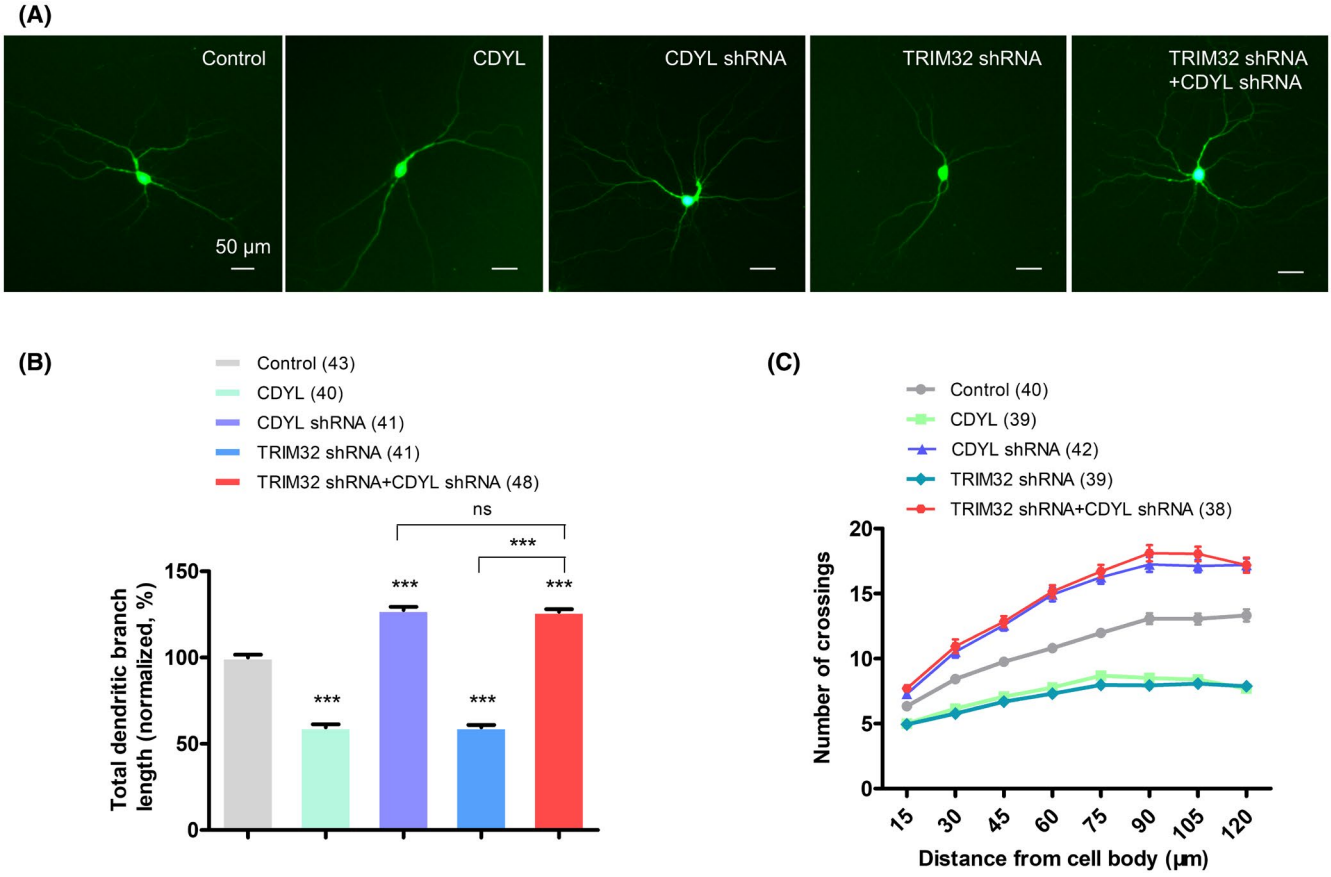

FIGURE 6 Tripartite motif-containing protein 32 (TRIM32) promotes dendritic complexity by regulating chromodomain Y-like (CDYL). (A) Representative images of hippocampal neurons transfected with the control vector, Flag-CDYL, Flag-shRNA, TRIM32 shRNA (T32-Sh3) or CDYL shRNA+TRIM32 shRNA plus GFP at 8 DIV. (B) Quantification of the total dendritic branch length of hippocampal neurons as shown in A. The methods used are shown in Figure 4B. The number labeled in the bracket indicates the number of neurons analyzed. (C) Sholl analysis of hippocampal neurons as shown in A. The number labeled in the bracket indicates the number of neurons analyzed

always degrade its substrates. TRIM32 can enhance the transcriptional activity and protein stability of retinoic acid receptor α (RARα) through ubiquitylation.[18] TRIM32 targets MITA for ubiquitylation through its E3 ubiquitin ligase activity, which promotes the interaction of MITA with TBK1.[32] To understand the function of TRIM32 more clearly, more substrates need to be identified. In this study, we identified CDYL as a novel target for TRIM32. CDYL can be ubiquitinated by TRIM32 in vivo and in vitro (Figure 2), and ubiquitinated CDYL was degraded by the 26S proteasome (Figure 3), thus promoting dendrite branching (Figures 4–6). In summary, our study defined a new biological function of TRIM32.

As the brain matures, dendrites form a highly complex branching architecture. Dendrite morphology is pivotal for many physiological and pathological conditions in the brain. Abnormal dendritic structure could impair circuit connection and information processing and eventually cause neurological diseases.[36–40] Dendrites are not fixed structures and have significant plasticity. Dendrite morphology can be reshaped in response to neuronal activity or in response to damage caused by disease or injury.[41,42] The molecular mechanisms underlying dendrite morphogenesis and related regulatory molecules are poorly understood. Here, we identified a new regulatory molecule of dendritic morphology, TRIM32 that can promote dendritic branching by degrading CDYL. Before this study, we found that CDYL can negatively regulate the development of dendritic branches by inhibiting the expression of BDNF. Hence, we provide a new member of this regulatory pathway involved in dendritic complexity. Our findings not only increase our understanding of the role of CDYL in neurodevelopment but may also provide targets for some neurological diseases.

ACKNOWLEDGMENTS

This work was supported by grant from the National Natural Science Foundation of China (31530028, 31720103908, 32030052 and 81821092) and the National

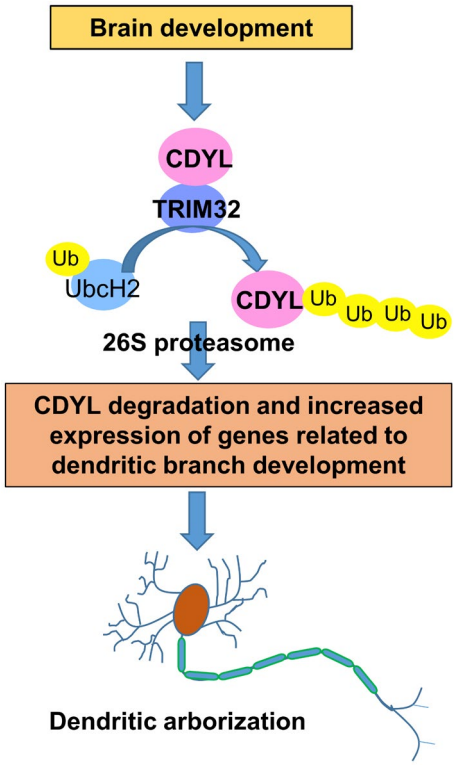

FIGURE 7 A working model of tripartite motif-containing protein 32 (TRIM32)-mediated chromodomain Y-like (CDYL) ubiquitylation and degradation involved in dendritic complexity. During brain maturation, CDYL is ubiquitinated and degraded by the E3 ubiquitin ligase TRIM32, leading to the activation of genes related to dendritic branch development and finally promoting dendritic branching

Key Technology Support Program of the Ministry of Science and Technology of China (2017YFA0701302). We thank Dr. Li Li in Peking University for providing the pXJ40-HA-Ub vector.

DISCLOSURES

The authors declare that there is no conflicts of interest.

AUTHOR CONTRIBUTIONS

Yun Wang and Lei Liu conceived and designed the research; Lei Liu and Ting-Ting Liu performed the research; Xiao-Qi Zhu and Guo-Guang Xie helped to perform the research; Yun Wang and Lei Liu analyzed the data; Yun Wang and Lei Liu wrote the paper.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

How to cite this article: Liu L, Liu T-T, Xie G-G, Zhu X-Q, Wang Y. Ubiquitin ligase TRIM32 promotes dendrite arborization by mediating degradation of the epigenetic factor CDYL. *FASEB J*. 2022;36:e22087. doi:10.1096/fj.202100031RR