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The E3 ubiquitin ligase HECTD3 regulates ubiquitination and degradation of Tara

Jian Yu [a], Jianping Lan [a], Yuanyuan Zhu [a], Xiaoxiao Li [a], Xiaoyu Lai [a], Yu Xue [b], Changjiang Jin [b,*], He Huang [a,*]

[a] *The First Affiliated Hospital of Zhejiang University Medical School, Hangzhou 310003, China*
[b] *Division of Cellular Dynamics, Hefei National Laboratory for Physical Sciences at the Microscale, University of Science and Technology of China, Huangshan Road, Hefei, Anhui Province 230027, China*

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Abstract

Tara was identified as an interacting partner of guanine nucleotide exchange factor Trio and TRF1. Tara is proposed to be involved in many important fundamental cellular processes, ranging from actin remodeling, directed cell movement, to cell cycle regulation. Yet, its exact roles required further elucidation. Here, we identify a novel Tara-binding protein HECTD3, a putative member of HECT E3 ubiquitin ligases. HECTD3 directly binds Tara *in vitro* and forms a complex with Tara *in vivo*. Overexpression of HECTD3 enhances the ubiquitination of Tara *in vivo* and promotes the turnover of Tara, whereas depletion of HECTD3 by small interfering RNA decreases Tara degradation. Furthermore, depletion of HECTD3 leads to multipolar spindle formation. All these findings suggest that HECTD3 may facilitate cell cycle progression via regulating ubiquitination and degradation of Tara.

Keywords: Tara; Ubiquitination; HECT E3 ubiquitin ligases; HECTD3; Degradation

The ubiquitin-proteasome system mediates the selective and time-dependent degradation of short-lived regulatory proteins, which are involved in a lot of critical cellular functions such as cell cycle progression, antigen presentation, transcriptional regulation, the induction of the inflammatory response, and apoptosis [1–4]. Protein degradation via this system occurs by two distinct and successive steps. Initially, target proteins are conjugated to the polypeptide ubiquitin through a multi-step reaction which is catalyzed by the sequential activity of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3); in the second step, the ubiquitin-conjugated proteins are recognized and degraded by proteasome. In the ubiquitin-proteasome system, the selectivity of substrate appears to be determined through the interaction between specific E3s and substrates [5]. Among the known ubiquitin ligases, HECT (homologous to E6-AP carboxyl terminus) E3 ubiquitin ligases are unique in that they participate directly in the chemistry of substrate ubiquitination reactions. All HECT E3 ligases contain a conserved C-terminal HECT domain which is homologous to the C-terminus of E6-AP [6] and a highly variable N-terminal domain which is responsible for substrate recognizing and binding [7].

Tara (Trio-associated repeat on actin) was originally identified as a Trio-binding protein [8]. Trio is a Dbl-homology guanine nucleotide exchange factor that regulates actin cytoskeletal reorganization, cell motility and cell growth [9]. Trio-deficiency causes mouse embryonic lethality associated with abnormal skeletal muscle and neural tissue development [10]. Interacting with Trio,

* Corresponding authors. Fax: +86 551 360 7141 (C.J.); fax: +86 571 8723 6706 (H.H.).
E-mail addresses: jincj@ustc.edu.cn (C. Jin), hehuang.zju@gmail.com (H. Huang).

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Tara is also implicated in regulating actin cytoskeletal organization. Ectopic expression of Tara alters actin cytoskeletal organization, promotes cell spreading, and enhances F-actin stability. Aside from interacting with Trio, our recent study showed that Tara also binds to TRF1 [11]. Considering the roles of TRF1 in telomere length control, telomeric ends shelter and cell cycle regulation [12–19], Tara may participate in telomere maintenance and/or mitotic regulation through interacting with TRF1.

Here, we performed a yeast two-hybrid screen and identified a novel Tara-binding protein HECTD3 (HECT domain-containing protein 3). Our studies show that HECTD3 interacts with Tara *in vitro* and *in vivo*. Overexpression of HECTD3 promotes the ubiquitination of Tara and accelerates the proteasomal degradation of Tara *in vivo*, wheras inhibition of HECTD3 increases Tara stability. Furthermore, depletion of HECTD3 leads to multipolar spindle formation. All these findings suggest that HECTD3 is an E3 ubiquitin ligase specifically recognizing Tara and facilitates mitotic progression by mediating degradation of Tara.

Materials and methods

Reagents. Anti-Tara polyclonal antibody was generated as described previously [20]. Other antibodies and reagents used in this study were as follows: mouse monoclonal anti-hemagglutinin (HA) antibody (Cell Signaling, Beverly, MA), mouse monoclonal anti-GFP antibody (BD Biosciences, San Diego, CA), rabbit monoclonal anti-MBP antibody (New England Biolabs Inc., Ipswich, MA), mouse monoclonal anti-FLAG antibody conjugated with horseradish peroxidase (HRP), mouse monoclonal anti-α-tubulin antibody, MG132, and Cycloheximide (CHX) (Sigma, St. Louis, MO), FITC-conjugated goat anti-mouse IgG and Rhodamine-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA).

Constructs. The cDNA encoding full-length human Tara [11] was constructed into pGBKT7, pEGFP-N1, p3XFLAG-myc-CMV-24 (Flag), and pET-22b(+) vectors. Full-length HECTD3 (GenBank Accession No. NM_024602) cDNA was purchased from OriGene (Rockville, MD) and then cloned into pGADT7, pEGFP-N3, p3XFLAG-myc-CMV-24, and pMal-C2 vectors. Flag-HECTD3 C539A mutant was prepared by PCR amplification method using two specific mutation primers (5′-AGTG GAAGACTCGGGCAGCG-3′ and 5′-GCCTCCAGCACCCTCTTC CTG-3′). All plasmid constructs were sequenced for verification.

Yeast two-hybrid. The yeast two-hybrid screen was carried out as described previously [21,22]. Interaction was verified by retransforming the candidate cDNAs including the full-length of HECTD3 back into AH109 yeast strain along with pGBKT7-Tara.

Recombinant protein. Expression and purification of recombinant His-Tara was carried out as described previously [20]. MBP (Maltose Binding Protein) and MBP-HECTD3 were expressed and purified by using amylose resin (New England Biolabs Inc., Ipswich, MA) according to the manufacturer's instructions.

Pull-down assay. His-Tara was purified and conjugated to Ni-NTA agarose beads (Qiagen, Valencia, CA). Purified MBP-HECTD3 or MBP was incubated with His-Tara conjugated Ni-NTA agarose beads at 4 °C for 2 h. Resultant agarose beads were washed three times with pre-cooled buffer (20 mM Tris–HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA) plus 1% Triton X-100 and three times with PBS. Proteins bound by agarose beads were fixed in Laemmli loading buffer, subjected to 10% SDS–PAGE, transferred to nitrocellulose membrane, probed with MBP monoclonal antibody, and finally developed with an ECL kit.

Cell culture and transfection. HeLa and 293T cells were cultured as described previously [22]. The transient transfection of plasmids and siRNA were performed by Lipofectamine™ 2000 or Oligofectamine™ reagent (Invitrogen, Carlsbad, CA), respectively, according to the manufacturer's recommendations.

Co-immunoprecipitation. For co-immunoprecipitation experiment, Flag-HECTD3 was co-transfected with GFP-Tara or GFP vector into 293T cells using calcium phosphate protocol. Thirty-six hours after transfection, cells were harvested and subjected to immunoprecipitation with anti-FLAG M2 affinity gel (Sigma, St. Louis, MO) as described previously [22].

Small interfering RNA (siRNA). For the siRNA studies, the siRNA duplex against HECTD3 was synthesized by Qiagen (Valencia, CA; Cat. No. SI00399336). As a control, either a duplex targeting cyclophilin or scramble sequence was used. After trial experiments using a series of concentrations and time course assays, treatment at 200 nM for 48 h was finally selected as the most efficient conditions for repressing HECTD3 protein.

Immunofluorescence. HeLa cells were grown on acid-treated glass coverslips. After siRNA transfection, immunofluorescence was carried out as described previously [23]. Slides were examined with a Zeiss Axiovert-200 fluorescence microscope, and images were collected and analyzed with Image-5 (Carl Zeiss, Germany). All images were processed with Adobe Photoshop 7.0 software.

Results

HECTD3 is a novel interactor of Tara

To identify interacting proteins of Tara, we conducted a yeast two-hybrid screen with Tara as a bait. Among the positive clones, we got a novel Tara-interacting protein encoding the N-terminal 75 amino acids of HECTD3, a putative E3 containing the HECT domain. To verify the interaction between HECTD3 and Tara, we cloned the full-length HECTD3 cDNA into pGADT7 vector and co-transformed AH109 yeast strain with pGADT7-HECTD3 and pGBKT7-Tara. As shown in Fig. 1A, the co-transformation assay confirmed HECTD3 interacts with Tara based in the yeast two-hybrid assay.

To validate the interaction between HECTD3 and Tara observed in our yeast two-hybrid assay, we carried out a pull-down assay to examine whether HECTD3 directly binds Tara in vitro. We expressed and purified MBP-HECTD3 and tested its ability to bind His-Tara that was conjugated to Ni-NTA agarose beads. As shown in Fig. 1B, His-Tara pulled down MBP-HECTD3 but not MBP. Therefore, the interaction between HECTD3 and Tara is physically direct.

To further confirm the interaction between HECTD3 and Tara, we performed co-immunoprecipitation experiments to test whether HECTD3 forms a complex with Tara *in vivo*. To this end, 293T cells were co-transfected with Flag-HECTD3 and GFP-Tara or GFP vector and then subjected to immunoprecipitation with anti-FLAG M2 affinity gel. Immunoblotting with GFP antibody showed that Tara is co-precipitated with HECTD3 (Fig. 1C). No GFP was recovered in the Flag immunoprecipitates, suggesting that the interaction between HECTD3 and Tara is specific and independent of the GFP tag.

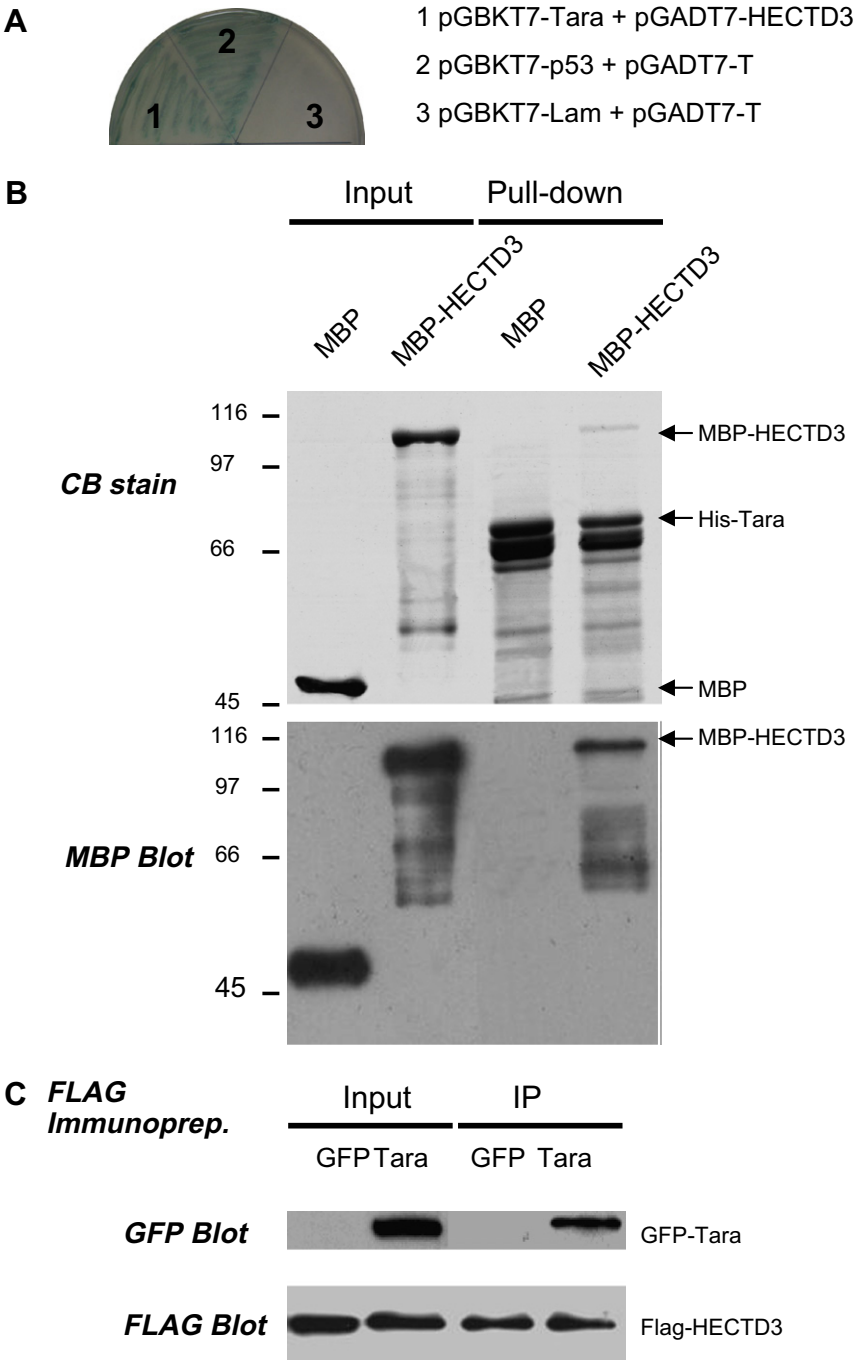

Fig. 1. Interaction of HECTD3 with Tara protein. (A) Interaction of HECTD3 with Tara in yeast. AH109 cells were co-transformed with indicated plasmids and then selected on supplemented minimal plates lacking tryptophan, leucine, histidine, and adenine. The interaction between HECTD3 and Tara was revealed through staining for β-galactosidase activity with X-α-Gal. (B) Direct physical interaction of HECTD3 with Tara determined by pull-down assay. His-Tara conjugated Ni-NTA agarose beads was used as affinity matrixes to absorb purified MBP-HECTD3 or MBP alone. Bound proteins were detected by immunoblotting analysis with MBP antibody. CB, Coomassie blue. (C) Co-immunoprecipitation of exogenously expressed HECTD3 and Tara. 293T cells were co-transfected with Flag-HECTD3/GFP-Tara or Flag-HECTD3/GFP and then subjected to immunoprecipitation with anti-FLAG M2 affinity gel, followed by immunoblotting analysis with GFP and Flag antibody.

HECTD3 promotes Tara ubiquitination in vivo

Our studies above demonstrated that HECTD3 interacts with Tara *in vitro* and *in vivo*. Given that the function of HECT E3 ligases is to target specific substrates for proteasomal degradation, we hypothesized that HECTD3 specifically recognize and ubiquitinate Tara, and thus target it for proteasome-mediated degradation.

To confirm this hypothesis, we first carried out *in vivo* ubquitination assay to determine whether Tara is ubiquitinated *in vivo*. 293T cells were co-transfected with Flag-Tara and HA-ubiquitin, lysed under denaturing conditions, and

then immunoprecipitated with anti-FLAG M2 affinity gel. Next, immunoblotting with FLAG and HA antibodies was performed to detect ubiquitinated Tara. As shown in Fig. 2A, overexpressed Tara was ubiquitinated *in vivo* and treatment of cells with the proteasome inhibitor MG132 caused a robust increase in Tara ubiquitination, suggesting that ubiquitinated Tara is a substrate for proteasome. No ubiquitinated Tara was detected in the absence of Flag-Tara, indicating the specificity of the *in vivo* ubiquitination assay.

Subsequently, we tested the effect of HECTD3 on Tara ubiquitination *in vivo*. 293T cells were co-transfected with Flag-Tara and HA-ubiquitin in the presence or absence of GFP-HECTD3. As expected, overexpression of HECTD3 caused an increase in Tara ubiquitination, and the Tara ubiquitination was further enhanced by MG132 treatment (Fig. 2B). These results indicated that HECTD3 can ubiquitinate Tara and thereby target it for proteasomal degradation.

Regulation of Tara degradation by HECTD3 in vivo

To further confirm our hypothesis that HECTD3 can specifically ubiquitinate Tara and target it for proteasomal degradation, we examined the effect of HECTD3 on Tara stability. To this end, we transfected HeLa cells with a fixed amount of GFP-Tara and variant amounts of Flag-HECTD3. As shown in Fig. 3A, the steady state of Tara was reduced by HECTD3 overexpression, indicating that HECTD3 can enhance the degradation of Tara. Using RNAi approach, we tested the effect of depletion of HECTD3 on Tara protein level. As shown in Fig. 3B and C, HECTD3 siRNA efficiently suppressed the expression of Flag-HECTD3 protein, and depletion of HECTD3 caused an increase of Tara protein level.

Subsequently, we determined whether the negative regulation of Tara protein level by HECTD3 is through modulating Tara degradation. To this end, GFP-Tara was co-transfected with Flag-HECTD3 or HECTD3 siRNA into HeLa cells. Next, protein translation was inhibited by cycloheximide treatment, and Tara protein levels were detected at the indicated times of treatment. As shown in Fig. 3D and E, overexpression of HECTD3 caused an increase in Tara degradation rate, wheras HECTD3 siRNA caused a decrease of Tara degradation rate.

Taken together, our results suggest that HECTD3 is an E3 for Tara that targets Tara for proteasomal degradation.

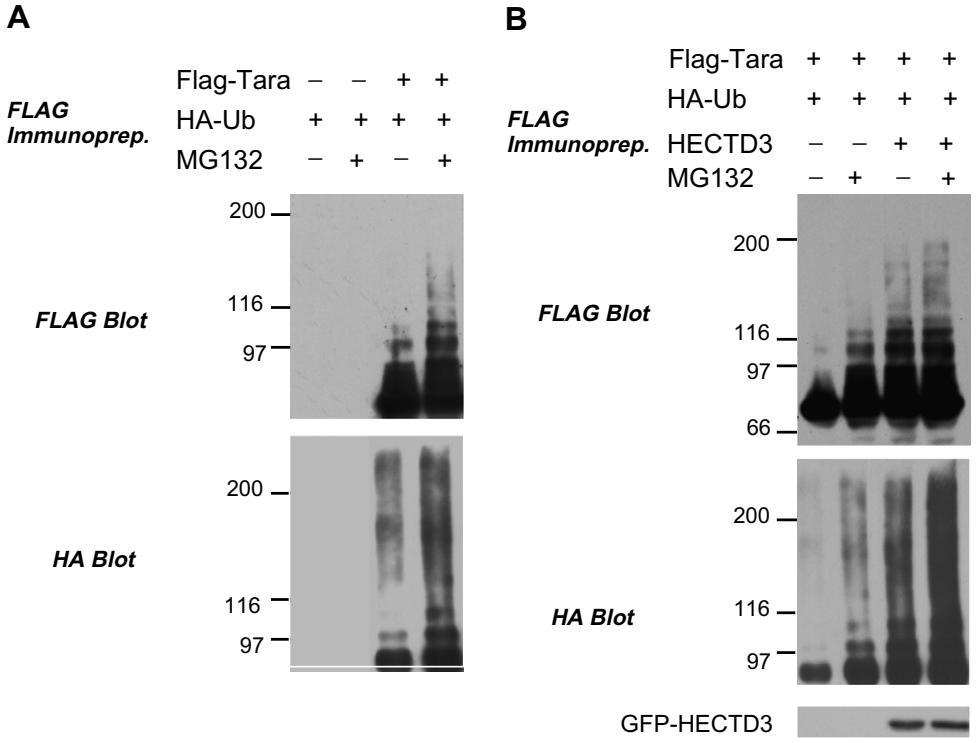

Fig. 2. Regulation of Tara ubiquitination by HECTD3 *in vivo*. (A) Ubiquitination of Tara *in vivo*. HA-ubiquitin was transfected alone or with Flag-Tara into 293T cells. Twenty-four hours later, the cells were treated with or without 25 μM proteasome inhibitor MG132 for another 6 h and then harvested. The cells were lysed under denaturing conditions (50 mM Tris–HCl, pH 7.4, 5 mM EDTA, 1% SDS, 10 mM DTT). After boiled at 95 °C for 5 min, the suspension was diluted with 0.9 ml nondenaturing lysis buffer (50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 5 mM EDTA, 1% Triton X-100) and clarified, and then immunoprecipitated with anti-FLAG M2 affinity gel, followed by immunoblotting analysis with FLAG and HA antibodies to detect ubiquitinated Tara. (B) Overexpression of HECTD3 enhances the ubiquitination of Tara *in vivo*. Flag-Tara and HA-ubiquitin were transfected with or without GFP-HECTD3 into 293T cells. Twenty-four hours later, the cells were treated with or without 25 μM proteasome inhibitor MG132 for another 6 h and then harvested. The cells were lysed under denaturing conditions and then immunoprecipitated with anti-FLAG M2 affinity gel. The ubiquitination of Tara was detected with FLAG and HA antibodies. The lower panel shows exogenous expression of HECTD3.

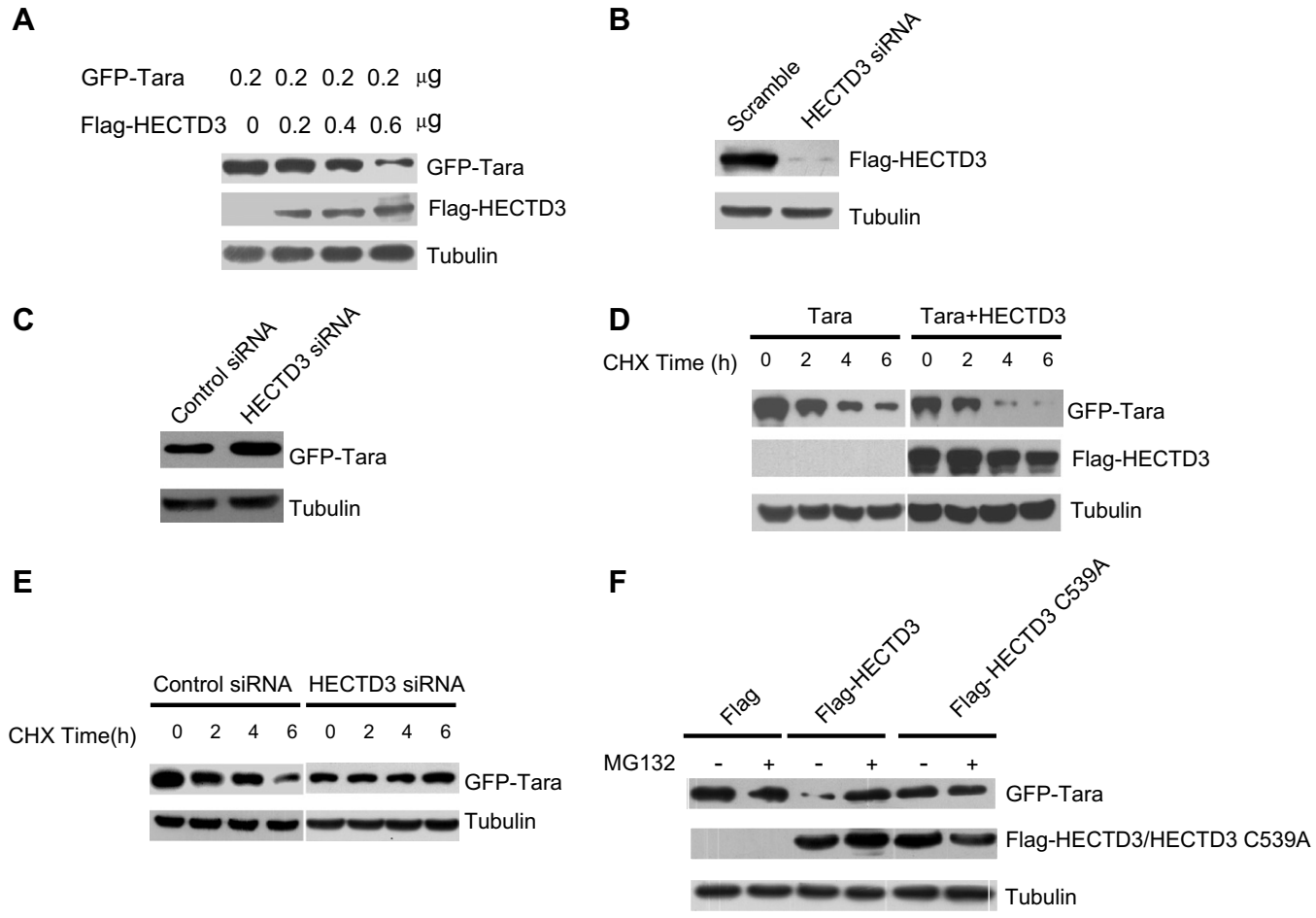

Fig. 3. Regulation of Tara degradation by HECTD3 *in vivo*. (A) Overexpression of HECTD3 decreases Tara protein level in a dose-dependent manner. A fixed amount of GFP-Tara and variant amounts of Flag-HECTD3 were co-transfected into HeLa cells as indicated followed by immunoblotting analysis with GFP antibody to detect Tara protein levels. The mid panel shows expression of HECTD3. The α-tubulin was used as the loading control. (B) Efficiency of HECTD3 siRNA treatment in HeLa cells. Aliquots of HeLa cells expressing Flag-HECTD3 were transfected with 200 nM siRNA oligonucleotide duplexes for HECTD3 or control (scrambled oligonucleotide) for 48 h and followed by immunoblotting analysis with FLAG antibody (upper panel) and α-tubulin antibody (lower panel). The analyses indicate the efficiency of the Flag-HECTD3 protein suppression. (C) Inhibition of HECTD3 increases Tara protein level. HeLa cells were transfected with oligonucleotides (control or siRNA for HECTD3) and GFP-Tara. Forty-eight hours later the cells were harvested and followed by immunoblotting analysis with GFP antibody to detect Tara protein levels. The α-tubulin was used as the loading control. (D) Overexpression of HECTD3 reduces the half-life of Tara. GFP-Tara was co-transfected with p3XFLAG-myc-CMV-24 vector or Flag-HECTD3 into HeLa cells. Twenty-four hours later, the cells were treated with cycloheximide (50 μg/ml) and harvested at indicated time points. The expression levels of GFP-Tara were determined by immunoblotting analysis with GFP antibody. The mid panel shows expression of HECTD3. The α-tubulin was used as the loading control. (E) Depletion of HECTD3 increases the half-life of Tara. HeLa cells were transfected with oligonucleotides (control or siRNA for HECTD3) and GFP-Tara. Forty-eight hours later, the cells were treated with cycloheximide (50 μg/ml) and harvested at indicated time points. The expression levels of GFP-Tara were determined by immunoblotting analysis with GFP antibody. The α-tubulin was used as the loading control. (F) Overexpression of HECTD3 but not HECTD3 C539A causes a decrease of Tara protein level. HeLa cells were co-transfected with GFP-Tara plus either p3XFLAG-myc-CMV-24 vector, Flag-HECTD3 or Flag-HECTD3 C539A. Twenty-four hours later, the cells were treated with or without 25 μM MG132 for another 12 h. The expression levels of GFP-Tara were determined by immunoblotting analysis. The mid panel shows expression of HECTD3 or HECTD3 C539A. The α-tubulin was used as the loading control.

The effect of HECTD3 on Tara stability depends on its E3 ligase activity

Previous studies showed that there is a conserved cysteine located 32–34 amino acids from the carboxyl end of the HECT domain. This cysteine serves as the site for ubiquitin transfer from E2 to the protein substrate via a transthiolation reaction catalyzed by the HECT E3 ligases [24]. According to the alignment of C-terminal sequences of various HECT domains of HECT E3 ligases and HECTD3 (Supplementary data, Fig. S1), we chose Cys539 as a mutation residue and constructed HECTD3 C539A mutant to test whether the effect of HECTD3 on Tara stability depends on its E3 ligase activity. We co-transfected HeLa cells with GFP-Tara plus either p3XFLAG-myc-CMV-24 vector, Flag-HECTD3 or Flag-HECTD3 C539A. As shown in Fig. 3F, in the absence of MG132, the protein level of Tara significantly decreased in cells expressing Flag-HECTD3, when compared with cells expressing Flag control or Flag-HECTD3 C539A. In the presence of

MG132, however, the levels of Tara became comparable in all cells. All the results implied that HECTD3 targets Tara for proteasomal degradation and this function depends on its E3 ligases activity.

Depletion of HECTD3 leads to multipolar spindle formation

Our recent discoveries that depletion of Tara results in multipolar spindle formation (manuscript in preparation) indicate that Tara may function in bipolar spindle formation and/or centrosomal stability. Given our results that HECTD3 targets Tara for proteasomal degradation, we hypothesized that HECTD3 may also be involved in mitotic regulation through mediating degradation of Tara. To confirm this, we transfected HeLa cells with siRNA for HECTD3 and stained with Tara and α-tubulin antibody. Consistent with our hypothesis, inhibition of HECTD3 leads to multipolar spindle formation (Fig. 4A and B). These results suggested that HECTD3 may provide a potential mechanism by which Tara protein function is regulated so as to facilitate cell cycle progression.

Discussion

Our previous studies demonstrated that Tara is a cell-cycle regulated protein and degraded through the ubiquitin-proteasome pathway (manuscript in preparation), but the exact mechanism remains unclear. Here, we report HECTD3, a putative E3 containing the HECT domain, is a novel interacting partner of Tara. HECTD3 directly

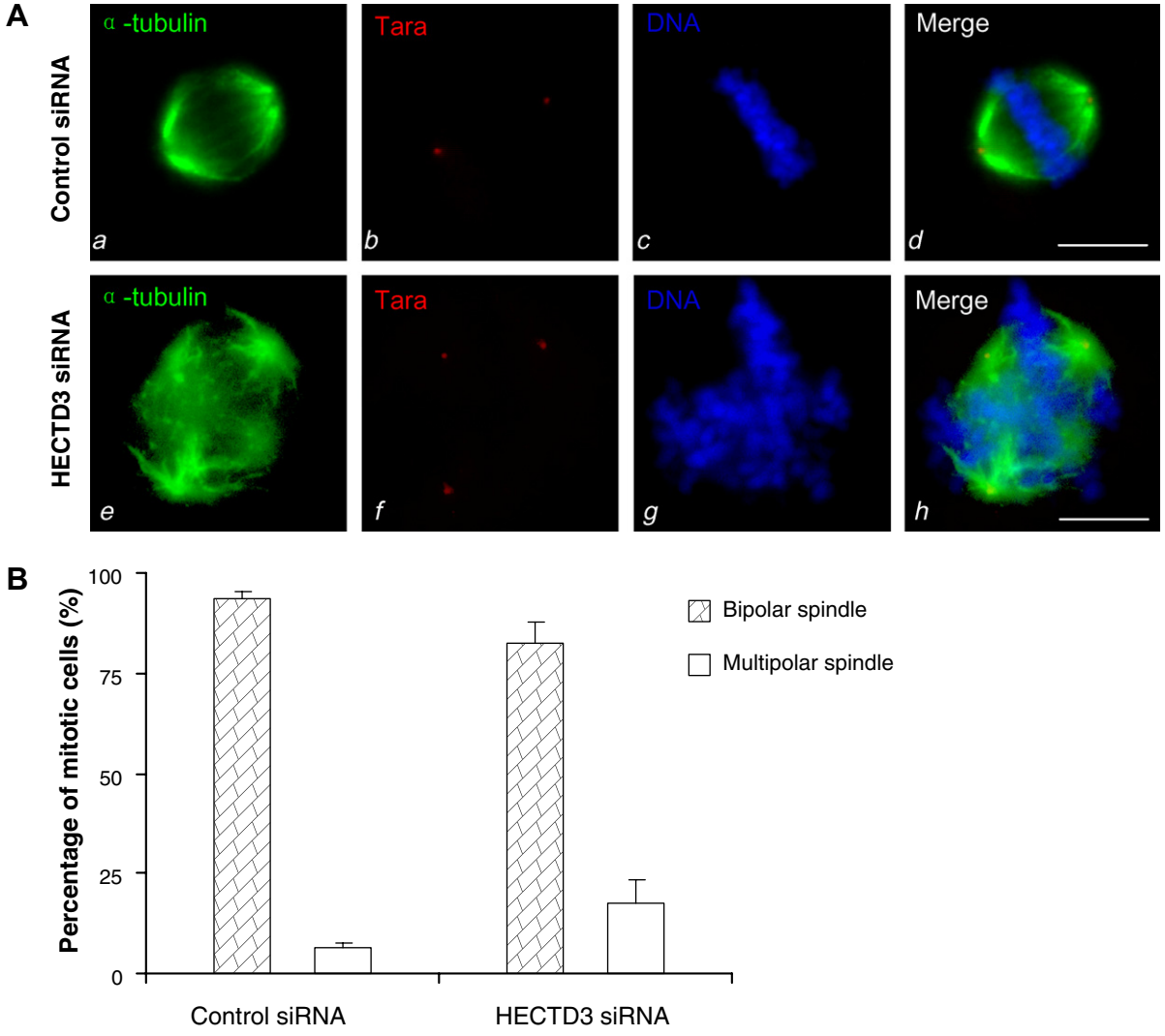

Fig. 4. Depletion of HECTD3 leads to multipolar spindle formation. (A) Inhibition of HECTD3 leads to multipolar spindle formation. HeLa cells were either mock-transfected (scramble) or transfected with HECTD3 siRNA oligonucleotide. Forty-eight hours post-transfection, the cells were extracted, fixed, and processed for immunocytochemistry. Optical images collected from HeLa cells transfected with mock (*a–d*) or HECTD3 (*e–f*) siRNA stained for anti-α-tubulin antibody (α-tubulin, green), anti-Tara antibody (Tara, red), DAPI (DNA, blue) and their merged images. Scale bars represent 10 μm. (B) Quantitative analyses of the spindle abnormality of HECTD3-depleted cells. We categorized the phenotypes as bipolar spindle and multipolar spindle. They were quantified and expressed as percentage of total mitotic cells. Values represent means ± SD from three separate experiments (at least 100 mitotic cells in each experiment scored). For interpretation of the references in colour in this figure legend, the reader is referred to the web version of this article.)

binds Tara *in vitro* and forms a complex with Tara *in vivo*. Further studies show that HECTD3 overexpression enhances the ubiquitination of Tara and decreases Tara stability *in vivo*, whereas depletion of HECTD3 increases Tara stability. Moreover, the effect of HECTD3 on Tara stability depends on its E3 ligase activity. Together, these findings suggest that HECTD3 can specifically ubiquitinate Tara and target it for proteasomal degradation.

In addition to Trio, Tara is a novel TRF1 interacting protein. TRF1 functions as a key molecule in connecting telomere maintenance and cell cycle control. Pin2/TRF1 translocates to centrosome upon the onset of mitosis [17,18]. Overexpression of TRF1 induces mitotic entry and apoptosis in cells with short telomeres [19]. Our recent studies showed that Tara traffics toward centrosome immediately upon nuclear envelope fragmentation and specifies the centrosomal localization of TRF1. Depletion of Tara results in multipolar spindle formation (manuscript in preparation). All these findings suggest that Tara may be involved in mitotic regulation through interacting with TRF1. Many centrosomal proteins are regulated in a cell-cycle dependent fashion, and the highly ordered cell cycle progression can be achieved by controlling the periodic expression and degradation of these centrosomal proteins [25–28]. Here, we show that HECTD3 targets Tara for proteasomal degradation and depletion of HECTD3 leads to multipolar spindle formation, indicating that HECTD3 may facilitate cell cycle progression by regulating the turnover of Tara. The exact mechanism underlying the spindle multipolarity induced by depletion of HECTD3 is not clear. Maybe the increase of Tara protein level by depletion of HECTD3 affects the localization of some proteins to the centrosome and leads to multipolar spindle formation.

Taken together, our studies demonstrate a critical role of HECTD3 in regulating cell cycle progression via controlling Tara's turnover.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2008.01.022.

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