Supporting Information

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SI Materials and Methods

Identification and Sequence Analysis of Ubiquitin-Related Family Genes in Chinese Amphioxus (*Branchiostoma belcheri tsingtauense*). The draft genome of *Branchiostoma belcheri* and the related analysis tools can be accessed at http://mosas.sysu.edu.cn/genome/. The following Pfam accession numbers were obtained from http://pfam.sanger.ac.uk/Software/Pfam.ubiquitin: PF00240; UBACT, PF02134; Ubiquitin_conjugation, PF00179; Zinc finger-C3HC4, PF00097; Zinc finger-RING–like, PF08746; U-box, PF04564; plant homeodomain PF00628; homologous to the E6-AP carboxyl terminus (HECT), PF00632; ovarian tumor, PF02338; ubiquitin C-terminal hydrolase, PF00443; DUSP, PF06337; JAB, PF01398; and Josephins, PF02099. Domain and gene identification were mainly performed with the HMMER2.0 plus SMART dataset (http://smart.embl-heidelberg.de). Previously unidentified protein architectures were validated by searching the EST and cDNA evidence from the EST dataset of *B. belcheri*, as well as the EST dataset and cDNA sets of *Branchiostoma floridae.*

Cell Culture and Biological Reagents. Adult Chinese amphioxus *B. belcheri tsingtauense* was obtained from Qingdao, China. HEK 293T and HeLa cells were maintained in DMEM (Invitrogen) supplemented with 10% FBS, 10 mM Hepes, and 2 mM L-glutamine. Recombinant human TNF-α expressed in *Escherichia coli* was obtained from PEPROTECH Asia. Antibody reagents were purchased from the indicated vendors: FLAG M2 monoclonal and Affinity Gel (Sigma–Aldrich), HA monoclonal (Roche) and anti-HA Affinity Gel (Sigma–Aldrich), and c-myc 9E10 monoclonal (Roche).

Cloning of bbt A20-Binding Inhibitor of NF-κB 1, bbt A20-Binding Inhibitor of NF-κB 2, bbtA20, and bbt NF-κB Essential Modulator cDNAs. Partial sequences of bbt A20-binding inhibitor of NF-κB 1 (bbtABIN1), bbtABIN2, bbtA20, and bbt NF-κB essential modulator (bbtNEMO) were cloned from *B. belcheri tsingtauense* intestinal cDNA by specific primer pairs. Subsequently, 5′-RACE and 3′-RACE were conducted according to the manufacturer's protocol using a GeneRACE Kit (Invitrogen) for full-length sequence cloning. Sequences of bbtABIN1, bbtABIN2, and bbtA20 have been deposited in the GenBank database under accession numbers KF006935, KF006936, and KF006937, respectively.

Section in Situ Hybridization. Section in situ hybridizations and acute immune challenges of adult amphioxus were performed according to our previous descriptions (1). Digoxigenin (Dig)-labeled sense and antisense probes for bbtABIN1, bbtABIN2, and bbtA20 were generated by the following primer pairs according to the Dig RNA Labeling Kit (Roche):

BbtABIN1-forward (F): CAGGAAGACAAGAGGCAGA
BbtABIN1-reverse (R): GAGTTCTCGGGATGACCTGA
BbtABIN2-F: GACGAGCTGACCGAGAGAAG
BbtABIN2-R: GGTCTATTCTGCGCTGTTGTT
BbtA20-F: CCAAGCTGAACCCAAATCCT
BbtA20-R: GACACTCTGGGATGATGCACT

Acute Immune Challenges of Adult Amphioxus and Real-Time PCR. Heated bacterial mixtures (10^5 cfu) of *Vibrio vulnificus* [Gram-negative (G^-)] and *Staphylococcus aureus* [Gram-positive (G^+)] were prepared for injection. The adult amphioxus was separated into three groups and treated with (*i*) DMSO as a negative control and (*ii*) MG132 or helenalin administered before inoculation with the bacterial mixtures. The animals were cultured in separate tanks, and the intestines from five individuals were collected at 0, 2, 4, 6, 8, and 12 h postinjection as a single sample. The following primer pairs were used:

BbtA20-F: GGAGGGAGGTGGAGGCTGGAA
BbtA20-R: TGGCTCTGGTGTGGTTGGAAT
Bbtβ-actinF: CCCTGTGCTGCTGACTGAG
Bbtβ-actinR: ACACGCCATCTCCAGAATCC

Expression Plasmids. Full-length cDNA fragments of bbtABIN1, bbtABIN2, bbtA20, and bbtNEMO were fused with Flag tag, HA tag, or Myc tag, respectively, and then inserted into pcDNA3.1 (Invitrogen). PCR fragments encoding for amino acids 1–300, 301–598, 1–350, 351–598, 1–470, and 471–598 of bbtABIN2 were fused with Flag tag, inserted into pcDNA3.1, and designated bbtABIN2-1 to bbtABIN2-6 (Fig. 3*B*). For the study of subcellular localization, the full-length bbtABIN1 and bbtABIN2 were inserted into pEGFP-N1 (Clontech). PCR fragments encoding for amino acids 1–390 and 391–907 of bbtA20 (designated bbtA1 and bbtA2, respectively) were fused with Flag tag, HA tag, or Myc tag, respectively, and then inserted into pcDNA3.1. The ORFs of bbtA20 with the catalytic cysteine at position 106 (C106) were replaced as alanine (designed as bbtAM1), or C584 and C589 were double-mutated into alanine (designed as bbtAM2), were fused with Myc tag, and subcloned into pcDNA3.1, respectively (Fig. 4*C*). The ORFs of WT amphioxus ubiquitin (designated bbtUb), of ubiquitin with Lys-48 and Lys-63 double-mutated into arginine (designated bbtUb-K48&63R), and of ubiquitin with all lysines mutated into arginine with the exception of Lys-48 or Lys-63 (designated bbtUbK48 and bbtUbK63, respectively) were fused with HA tag and subcloned into pcDNA3.1. Point mutations were made by site-directed PCR mutagenesis and verified by DNA sequence analysis.

FLAG or HA Elutions and Protein Purifications. For FLAG or HA elutions, HEK 293T cells were transfected with the indicated Flag-tagged or HA-tagged constructs as described above. After 24–48 h, cells were collected in PBS and Dounce-homogenized in a hypotonic lysis buffer containing 10 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris (pH 7.5), and Complete protease inhibitor mixture (Roche). Lysates were cleared by centrifugation and immunoprecipitated with anti-Flag Affinity Gel (Sigma–Aldrich) or anti-HA Affinity Gel 4 h to overnight. Immunoprecipitates were washed once with wash buffer 1 [20 mM Hepes (pH 7.9), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 200 mL/L glycerol, Complete protease inhibitor mixture] and four times with wash buffer 2 [20 mM Tris (pH 7.4), 150 mL/L glycerol, 0.2 mM EDTA, 300 mM NaCl, 0.1% Nonidet P-40, Complete protease inhibitor mixture] and rotated at least 2 h in wash buffer 2. Samples were eluted with 5 μg/μL 3× FLAG peptide (Sigma–Aldrich) or influenza HA peptide (Sigma–Aldrich) according to the manufacturer's instructions.

In Vitro Deubiquitination Assays. Flag-bbt receptor-interacting serine/threonine protein kinase 1b (RIP1b) immunoprecipitates were washed three times with PBS and twice with buffer containing 50 mM Hepes at pH 8.0 and 0.1% Nonidet P-40. HA-tagged bbtA20 (up to 1 μg) and substrates (up to 1 mM) were combined in a buffer containing 50 mM Hepes at pH 8.0, 0.1% Nonidet P-40, and 3 mM DTT and were incubated at 37 °C for

150 min (free N-terminal His-tagged K63-linked polyubiquitin chain; Boston Biochem) or for the indicated time (Flag-bbtRIP1b substrate). Samples were subsequently prepared for immunoblot analysis.

Ubiquitin Chain-Binding and Pull-Down Assay. Flag-bbtABIN2 immunoprecipitates were washed three times with pull-down buffer [PDB; 150 mM NaCl, 50 mM Tris (pH 7.5), 5 mM DTT, 0.1% Nonidet P-40]. The washed beads were then incubated with 1.5 μg of individual chain in 50 μL of PDB plus BSA (0.5 mg/mL) overnight at 4 °C. The beads were washed five times with PDB, mixed with 4× SDS loading buffer, and boiled for 2 min for immunoblot analysis.

Luciferase reporter assays, immunofluorescence imaging, and coimmunoprecipitation were conducted as described in our previous study (2). Values were expressed as mean relative stimulations for a representative experiment from three separate experiments, with each performed in triplicate. Values were considered to be significant when $P < 0.05$. The pNF-κB-Luc plasmid was purchased from Stratagene, and Renilla expression vector pRL-TK was purchased from Promega.

1. Yuan S, et al. (2010) Amphioxus SARM involved in neural development may function as a suppressor of TLR signaling. *J Immunol* 184(12):6874–6881.

2. Li J, et al. (2011) Functional conservation and innovation of amphioxus RIP1-mediated signaling in cell fate determination. *J Immunol* 187(8):3962–3971.

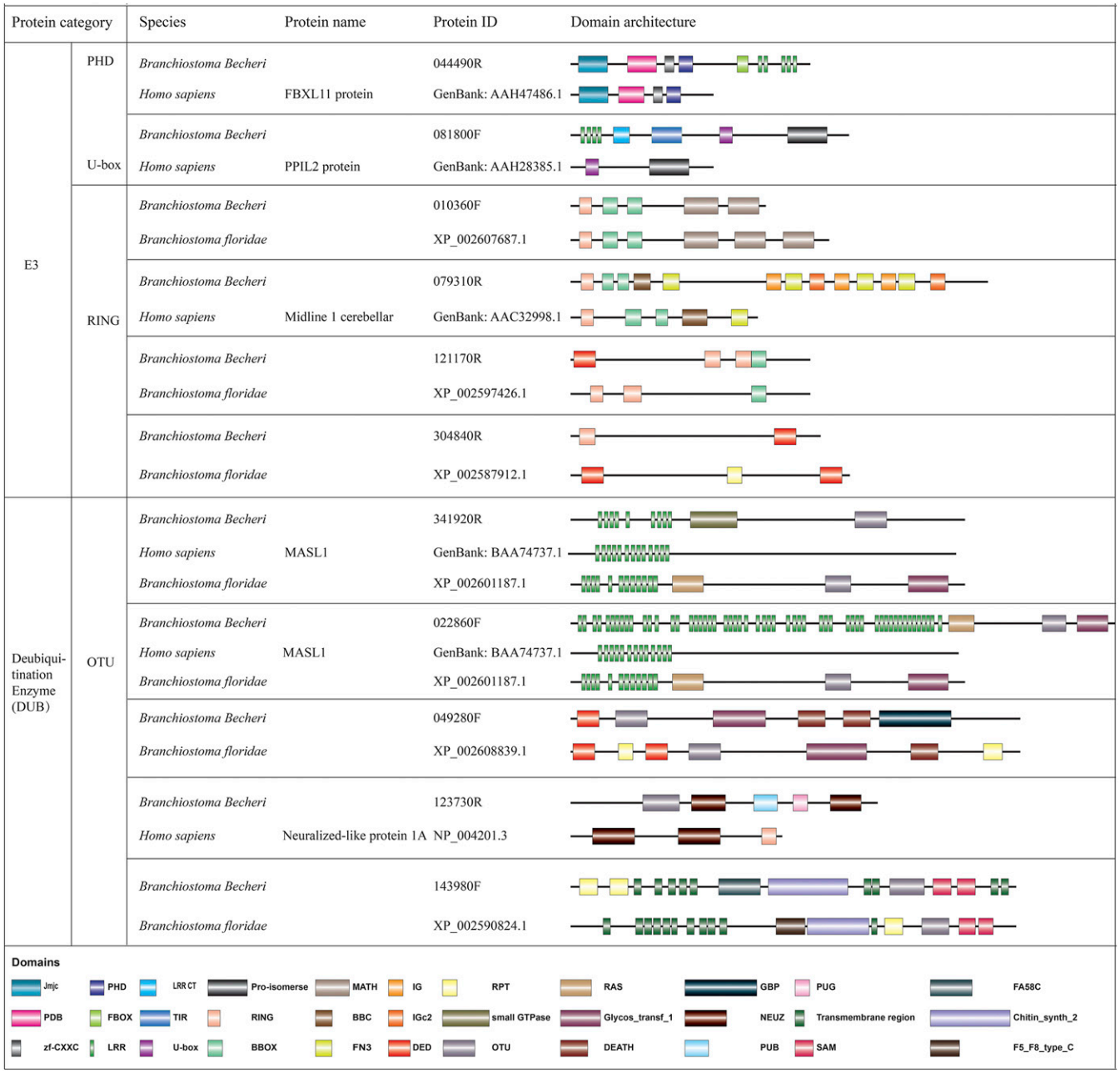

Fig. S1. Putative E3s and deubiquitinating enzymes (DUBs) with previously unidentified domain architecture in amphioxus. Ring fingers linked with additional death effector domain (DED) and ovarian tumor (OTU) domain linked with additional DED or death domain (DEATH) or with leucin-rich repeat (LRR) were found in amphioxus E3s and DUBs. BBC, B-box C-terminal; BBOX, B-box–type zinc finger; FBOX, F-box domain; FN3, fibronectin type 3; GBP, guanylate-binding protein; IG, immunoglobulin; Jmjc, Jumonji C; LRRCT, leucine rich repeat C-terminal; MATH, meprin and TRAF homology; NEUZ, domain in neuralized proteins; OTU, ovarian tumor; PDB, in PDBSUM for further annotation; PHD, plant homeodomain; PUB, domain in proteins involved in the ubiquitin/proteasome-related pathway; PUG, domain in peptide N-glycanases and other putative nuclear proteins; RAS, Ras subfamily of RAS small GTPases; RPT, repeat domain; SAM, sterile alpha motif; TIR, Toll/IL-1R homology; zf-CXXC, CXXC zinc finger.

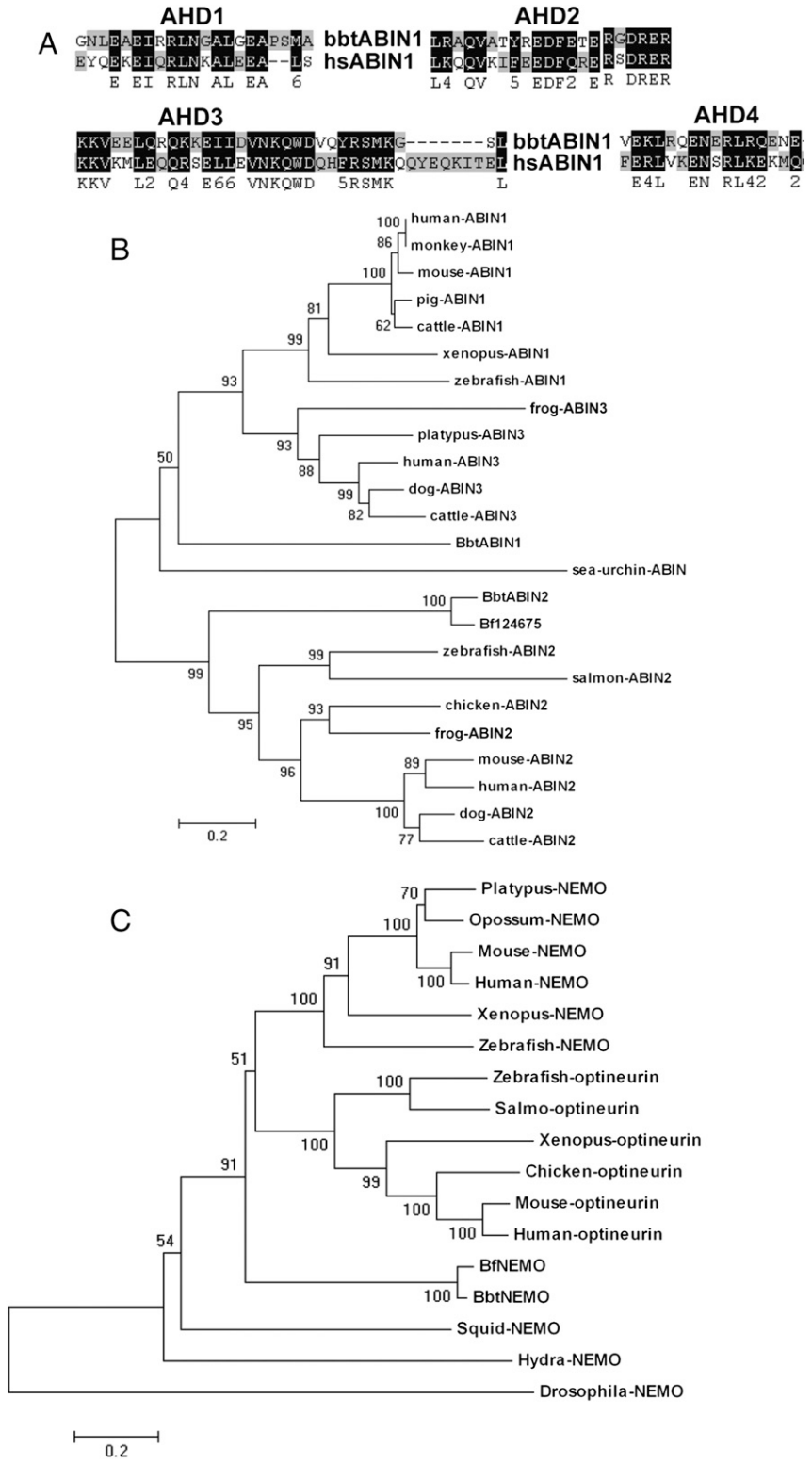

Fig. S2. (*A*) Sequence alignment of the ABIN homology domains (AHDs) between human ABIN-1 and bbtABIN1. (*B*) Phylogenetic analysis of bbtABINs. (*C*) Phylogenetic analysis of bbtNEMO. Protein sequences of ABINs and NEMOs were obtained from the GenBank database and were first aligned using ClustalX 1.83 and manually corrected using GeneDoc. The neighbor-joining tree was then obtained using the routine in MEGA4 with 1,000 bootstrap tests.

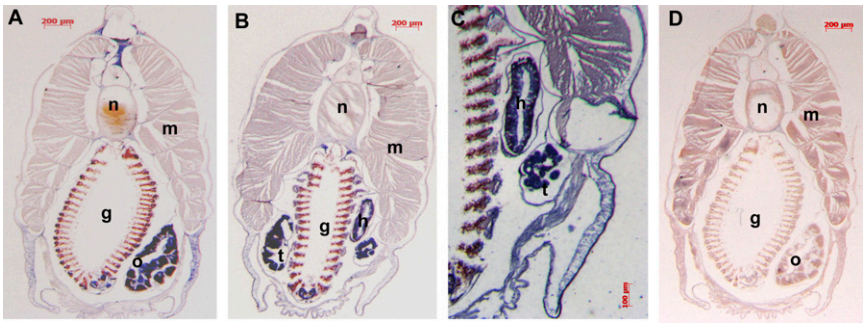

Fig. S3. Tissue distribution of bbtABIN1. (*A* and *B*) Section in situ hybridization analysis of bbtABIN1 antisense probe showed that transcripts of bbtABIN1 were abundant in hepatic cecum, testis, and ovary. (*C*) Macroscopic view of the hybridization signals of bbtABIN1 in hepatic cecum. (*D*) Negative control of bbtABIN1 using the sense probe. g, gill slit; h, hepatic cecum; m, muscle; n, notochord; o, ovary; t, testis.

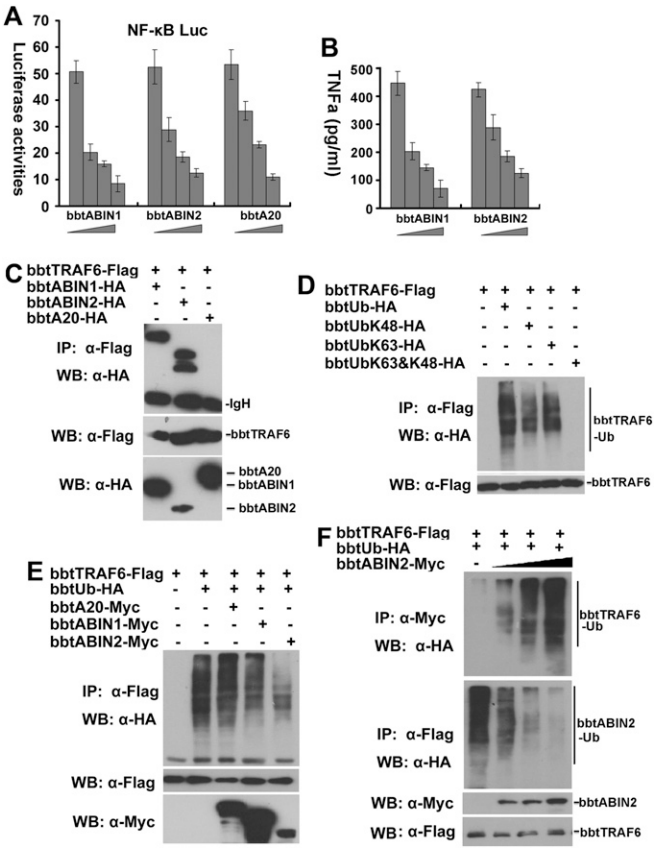

Fig. S4. (*A*) Luciferase reporter assays showed that all bbtABIN1, bbtABIN2, and bbtA20 can attenuate the TNF-α–induced NF-κB activation in a dose-dependent manner in HEK 293T cells. (*B*) ELISA assay showed that both bbtABIN1 and bbtABIN2 can attenuate bbt TNF receptor-associated factor 6 (bbtTRAF6)-mediated induction of TNF-α in a dose-dependent manner in HEK 293T cells. (*C*) Both bbtABIN1 and bbtABIN2 can coprecipitate with bbtTRAF6 by coimmunoprecipitation (IP) analysis. (*D*) Ubiquitination assay showed that both K63-linked and K48-linked polyubiquitin chains can be built on bbtTRAF6. (*E*) When bbtTRAF6 was coexpressed with bbtABIN2, but not with bbtNEMO or bbtABIN1, a low efficacy of K63-linked ubiquitination on bbtTRAF6 was observed. (*F*) In vivo ubiquitination showed that bbtABIN2 can compete with bbtTRAF6 for the K63-linked polyubiquitin chains. WB, Western blot.

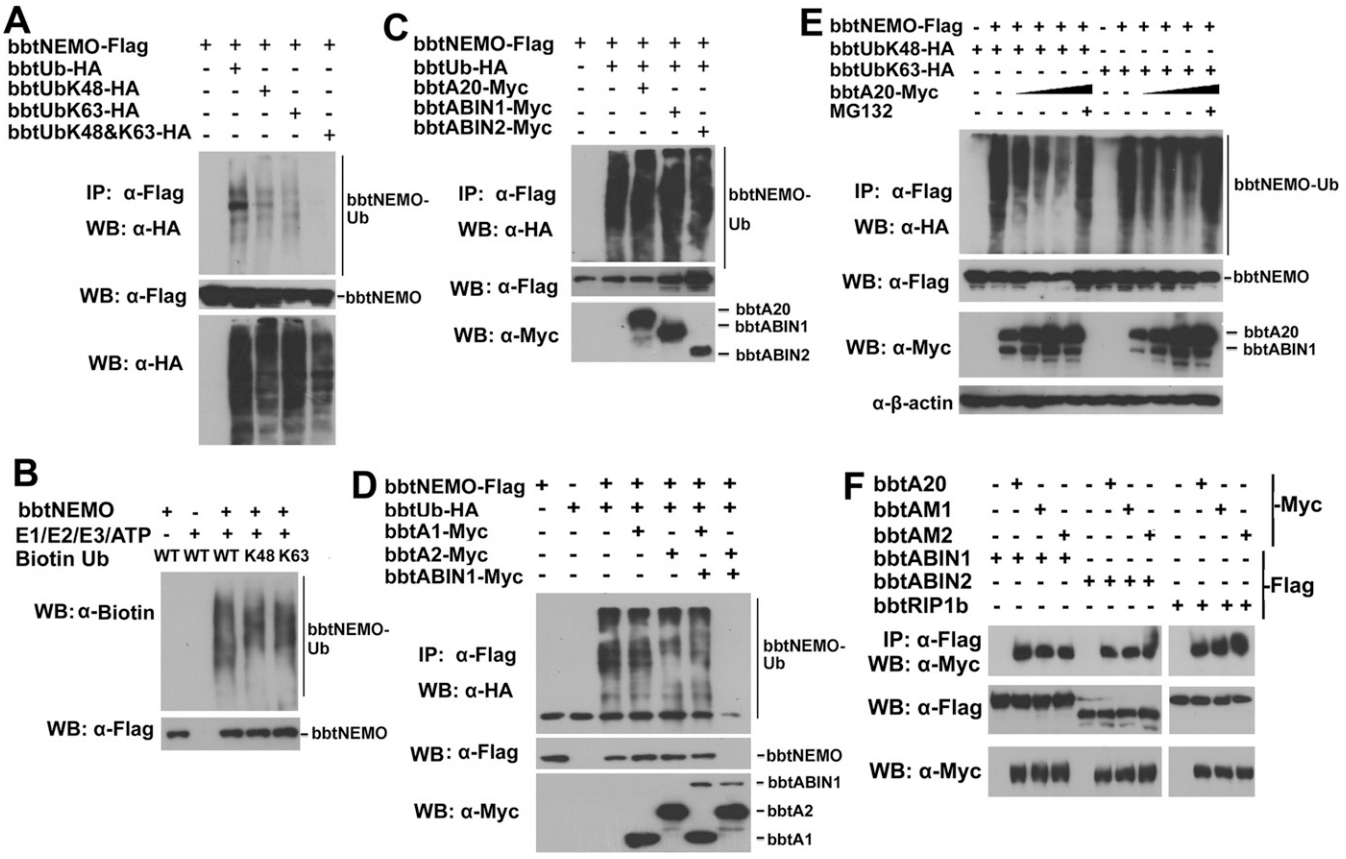

Fig. S5. (*A*) In vivo ubiquitination showed that both K48-linked and K63-linked polyubiquitin chains can be built on bbtNEMO. (*B*) In vitro ubiquitination showed that both K48-linked and K63-linked polyubiquitin chains can be built on bbtNEMO. (*C*) Alone, bbtABIN1, bbtABIN2, or bbtA20 could not promote the deubiquitination or degradation of bbtNEMO. (*D*) In vivo ubiquitination showed that in the presence of bbtABIN1 and bbtUbK48, bbtA2 [the zinc fingers (ZnFs) only] can mediate the degradation of bbtNEMO. (*E*) In vivo ubiquitination assay showed that in the presence of bbtABIN1, bbtA20 can induce the proteasome-dependent degradation of bbtNEMO. (*F*) Mutations of bbtA20 in OTU and ZnF4 did not affect its binding with bbtABINs and bbtRIP1b.

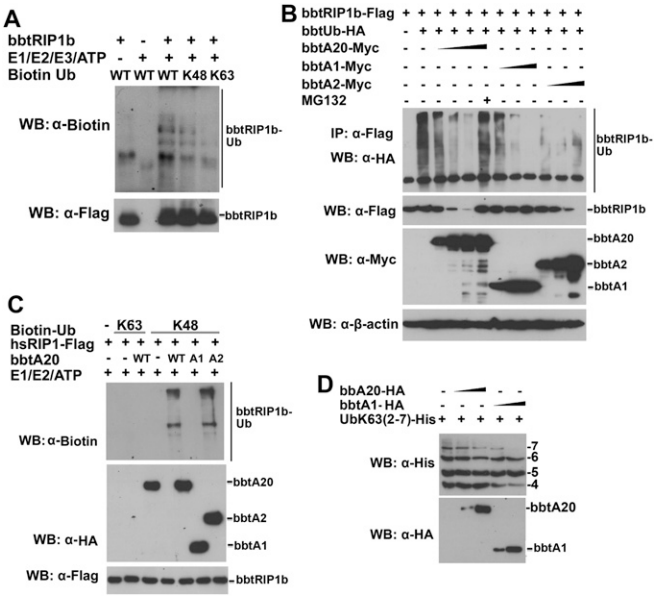

Fig. S6. (*A*) In vitro ubiquitination showed that both K48-linked and K63-linked polyubiquitin chains can be built on bbtRIP1b. (*B*) In vivo ubiquitination assays showed that bbtA1 (the OTU only) is responsible for deubiquitination of bbtRIP1b, whereas the bbtA2 (the ZnFs only) mediates the degradation of bbtRIP1b. (*C*) In vitro ubiquitination assay showed that bbtA20 can catalyze the K48-linked ubiquitin chains on bbtRIP1b. (*D*) In vitro deubiquitination assay showed that bbtA20 can deubiquitinate the K63-linked ubiquitin chains.

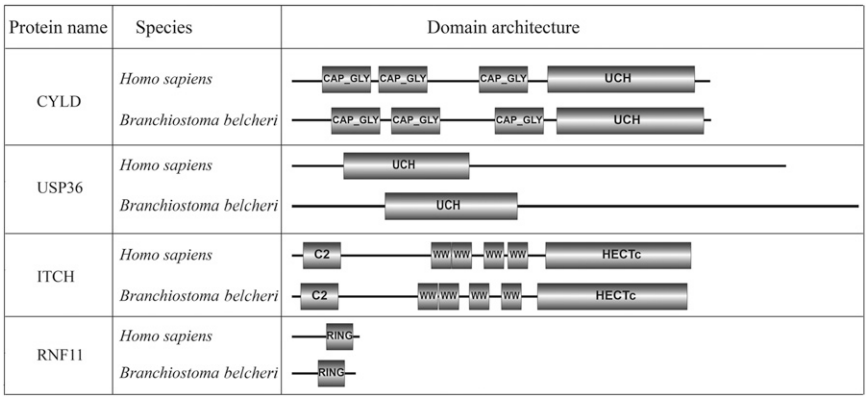

Fig. S7. Domain architectures of cylindromatosis (CYLD), ubiquitin-specific protease 36 (USP36), itchy E3 ubiquitin protein ligase (ITCH), and ring finger protein 11 (RNF11) between *Homo sapiens* and amphioxus (*B. belcheri*). CAP_GLY, cytoskeleton-associated proteins (CAPs) glycine-rich; HECTc, homologous to the E6AP carboxyl terminus; RING, really interesting new gene; UCH, ubiquitin C-terminal hydrolase; WW, also known as the WWP or rsp5 domain.

Table S1. Gene modules related to ubiquitination in genomes among species

Protein names		Fruit fly	Amphioxus	Zebrafish	Human being
Ubiquitin		24	45	47	63
UBA_E1		1	1	putative 2	2
UBC_E2		33	42	45	41
E3s	HECT	14	25	38	29
	RING	132	389	395	362
	U-box	5	9	15	10
	PHD	44	69	99	99
DUBs	USP	22	41	50	74
	UCH	2	5	7	6
	OTU	7	32	14	15
	MJD	1	2	4	4
	JAMMs	10	12	11	12

DUBs, deubiquitinating enzymes; JAMMs, JAB1/MPN/MOV34 metalloenzymes; MJD, Josephins; OTU, ovarian tumor; PHD, plant homeodomain; RING, really interesting new gene; UBA, ubiquitin-associated; UBC, ubiquitin conjugating; UCH, ubiquitin C-terminal hydrolases; USP, ubiquitin-specific protease.