ANKIB1 Functions as a Partner of E3 ubiquitin ligase

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Research Article

Keywords: ANKIB1, E3 ligase, RBR, ubiquitination, ubiquitin-conjugating enzyme

Posted Date: February 24th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-232896/v1

ANKIB1 functions as a partner of E3 ubiquitin ligase

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Abstract

ANKIB1 is an RBR domain-containing protein. It has been classified as an E3 ubiquitin ligase for a long time. However, little is known about its E3 ligase activity and related ubiquitin-conjugating enzymes (E2s). We investigated the expression pattern, cellular localization, and brain distribution of ANKIB1. Immunoblotting showed that human cancer cell lines had different expression patterns of ANKIB1 with virus-transformed immortal lymphocytes and human brain. Immunocytofluorescence analysis indicated that ANKIB1 was mainly localized in the cytoplasm, and immunohistochemistry staining demonstrated that ANKIB1 was highly expressed in the rat brain cortex but seldom expressed in the basal ganglia. We then performed coimmunoprecipitation, *in vitro* and *in vivo* ubiquitination assay to determine whether ANKIB1 functions as an E3 ligase. A panel of class I and class III E2s were tested, of which UbcH8, UbcH10, and UbcH13 were confirmed to interact with ANKIB1. Moreover, ANKIB1 showed E3 ligase activity both *in vivo* and *in vitro*, but it promoted protein ubiquitination more efficiently *in vivo*. These data suggest that ANKIB1 acts as a partner of an E3 ligase complex, and may play important roles in regulating tumorigenesis and brain cortex function.

Keywords ANKIB1 · E3 ligase · RBR · ubiquitination · ubiquitin-conjugating enzyme

Declarations

Funding This work was supported by the National Natural Science Foundation of China (grant no. 81601378), the Foundation and Frontier Technology Research Program of Henan Province, China (grant no. 152300410120), and the Scientific Research Fund of Xinxiang Medical University (2013ZD103).

Conflict of interest The authors declare no conflict of interest.

Availability of data and materials All data and materials generated or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions Bohong Xie contributed to the Founding acquisition, bioinformatic analysis, E2s interaction, and Northern Blotting. Zhenling Ma was responsible for the plasmid construction and ubiquitination analysis. Zishan Yang performed the immunocytofluorescence and immunohistochemistry assay. Yuanbo Li contributed to the expression and purification of recombinant proteins. Shirao Liu contributed to the plasmid construction. Haijie Yang, Lei Wang, Binfeng Cheng, and Zhiwei Feng provided suggestions on experimental design and data analysis. Yunfei Xie was responsible for Founding acquisition, conceptualization, experimental design, data analysis, and manuscript writing. The manuscript is approved by all authors for publication.

Ethics approval The current study was approved by the Ethics Committee of Xinxiang Medical University. All animal procedures were following the Animal Management Rules of the Ministry of Science and Technology of the People's Republic of China for experimental care and use of animals.

Abbreviations

AA	amino acid
ANK	ankyrin repeat
ANKIB1	ankyrin repeat and IBR domain-containing protein 1
ARA54	androgen receptor-associated protein 54
ARIH1	Ariadne-1 homolog
ARIH2	Ariadne-2 homolog
CRL	Cullin-RING ligase
CUL9	Cullin-9
DAPI	4',6-diamidino-2-phenylindole
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GST	glutathione S-transferase
HECT	homologous to E6-AP carboxy terminus
HHARI	human homolog of Ariadne
IBR	in-between RING
IPTG	isopropyl-β-D-thiogalactopyranoside
mAb	monoclonal antibody
Parc	p53-associated, Parkin-like cytoplasmic protein
PBS	Phosphate buffered saline
PBST	PBS with 0.1% Triton X-100
PARK2	Parkinson disease protein 2
RBR	RING-IBR-RING
RING	really interesting new gene
SOCS	suppressors of cytokine signaling
TRIAD1	two RING fingers and DRIL [double RING finger linked] 1
Ub	ubiquitin
Ubc	ubiquitin-conjugating enzyme
UIM	ubiquitin-interacting motif

Introduction

The ankyrin repeat (ANK) is one of the most widespread protein domains. Nearly 800 human proteins have been found to contain ANK domain (SMART's nrdb database). ANK is also the most common domain to mediate protein-protein interaction in nature. Unlike other protein-protein interaction motifs or domains such as LRR (leucine-rich repeat) or SH3, ANK domain can not recognize any specific amino acid sequence or structure. Instead, various copies of ANK are packed together with either themselves or in conjunction with other domains to form the underlying architecture of an extended, modular interface exposed to proteins for binding. Other functional domains that can co-exist with ANK domain include the F-box, SOCS-box, Zn-finger domain, ion-transport domain, and protein kinase domain, etc [1]. ANK domain can offer proteins a range of advantages in stability, binding affinity, and rigid structure, etc. These make it quite useful in designing recombinant ankyrin repeat proteins for research and clinical application [2]. ANK-containing proteins play a variety of roles ranging from cytoskeleton organization, cell cycle control, genomic stability, apoptosis, vesicular trafficking, autophagy, and inflammatory response, etc [3-6].

The ubiquitin (Ub) system plays a key role in regulating protein function and degradation. Protein ubiquitination is mediated by a three enzymatic cascade consisting of ubiquitin-activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin ligase (E3). E3 ligases have been divided into three families depending on the structure and mechanism: the RING-finger E3s, HECT-type E3s, and RBR-type E3s [7]. The most prevalent ubiquitin ligases are RING-type which can be further categorized as single peptide RING E3s, U-box (a modified RING finger domain) E3s, and CRLs [8]. As an emerging group of ubiquitin ligases, RBR-type E3s are characterized by the containing of a RING-IBR-RING supradomian. They have been defined as hybrids of RING and HECT E3s in mechanism [9]. Only 14 human RBR proteins have been identified to date [10, 11]. The best characterized RBR protein is Parkin/PARK2, which plays a key role in the autosomal recessive Parkinson disease [12]. The largest RBR cohort is the Ariadne subfamily, which includes ARIH1/HHARI, ARIH2/TRIAD1, CUL9/PARC, and ANKIB1 (originally named KIAA1386). All the Ariadne RBRs have been well characterized except ANKIB1.

ANKIB1 was first discovered in the human brain [13]. It has been considered as an E3 ligase for a long time due to its structure. But no experimental evidence has been raised to support the E3 activity of ANKIB1 to date, nor have ANKIB1-associated ubiquitin-conjugating enzymes been determined. Most recently, circular ANKIB1 RNA (circ-ANKIB1) was found to play a critical role in depressing Schwann cell proliferation following sciatic nerve injury [14]. Meanwhile, circ-ANKIB1 was reported to promote osteosarcoma cell growth and invasion by upregulating miR-19b expression [15]. Nevertheless, little is known about the function of ANKIB1 protein except that its UIM may participate in protein ubiquitination [16, 17]. Therefore, this study focuses on identifying Ubcs that can interact with ANKIB1 and clarifying the E3 ligase activity of ANKIB1. Moreover, the structural homology and dissimilarity between ANKIB1 and other RBR proteins, as well as the subcellular localization of ANKIB1 and its distribution in the rat brain, were explored.

Materials and methods

Plasmid Construction

The recombinant plasmids pCMV-Myc-ANKIB1, pCMV-Myc-ANKIB1(1-480), and

pGEX-5X-ANKIB1(1-480) have been described previously [18, 19]. pGEX-4T-UbcH5B, pET-28a-UbcH7, and pGEX-4T-UbcH8 were generous gifts from professor Brian Kuhlman (Department of Biochemistry and Biophysics, University of North Carolina). These vectors were used as templates to amplify the coding region of ANKIB1^{RBR} (amino acid residues 330~570 of human ANKIB1), UbcH5B, UbcH7, or UbcH8 by PCR. Then the target fragments were ligated with pET-28a (Novagen) or pFLAG-CMV-2 (Sigma-Aldrich) respectively to construct pET-28a-ANKIB1^{RBR}, pFLAG-CMV-UbcH5B, pFLAG-CMV-UbcH7, and pFLAG-CMV-UbcH8 by double restriction endonuclease digestion strategy. pRK5-HA-Ub was a gift from Ted Dawson (Addgene plasmid #17608). pET22a-UbcH6 was a gift from Peter Howley (Addgene plasmid #8653). pET-21b-Ub and pFLAG-CMV-UbcH6 were constructed by using the twin PCRs cloning method [20]. pCMV-FLAG-UbcH10 and pCMV-FLAG-UbcH13 were purchased from Sino Biological (Beijing, China). Primers used for PCR are listed in Table 1.

Expression and Purification of recombinant proteins

His_6- or GST-tagged recombinant proteins were expressed in *E. coli* BL21 (DE3). His_6-ANKIB1^{RBR} was induced with 0.3 mM IPTG (Sangon, China) at 20℃ for 6 h. His_6-Ub, GST-ANKIB1(1–480), GST-UbcH5B, GST-UbcH7, and GST-UbcH8 were induced with 0.1 mM IPTG at 20℃ for 6 h. The proteins were purified by Ni-NTA (Qiagen) or Glutathione Sepharose 4B (GE Healthcare) affinity chromatography. GST-tagged Ubcs were further treated with thrombin to cleave GST tag.

Cell Culture and Transfection

Human embryonic kidney HEK293T cells, human cervical carcinoma Hela cells, human hepatocellular carcinoma cells HepG2 and SMMC-7721, and human neuroblastoma SH-SY5Y cells were cultured in DMEM supplemented with 2 mM L-glutamine and 10% FBS (Tianhang Biotech, China). The human T-lymphotropic virus Type 1 positive human T cells MT-2 and MT-4, Epstein-Barr virus-transformed human B lymphoblast cells IM-9, and mouse myeloma sp2/0 cells were cultivated in RPMI 1640 medium supplemented with 10% FBS. Cells were maintained in an incubator at 37 ℃ in a humidified atmosphere containing 5% CO_2. For transfection, HEK293T Cells were seeded in a 6-well or 60 mm plate and transfected with Xfect transfection reagents (Clontech) according to the manufacturer's protocol.

Northern Blotting Analysis

Using plasmid pCMV-Myc-ANKIB1(1-480) as a template, ANKIB1-specific cDNA probe corresponding to c.21-994 of the ANKIB1 mRNA (NM_019004.2: 657-1630) was generated and labeled by utilizing PCR DIG Probe Synthesis Kit (Roche) and primer pair ANK-NBF/ANK-NBR. 10 μg total RNA isolated from the SH-SY5Y cells was separated on 1% formaldehyde-containing agarose gel and transferred to a Hybond-N^+ nylon membrane (GE Healthcare). Following baking at 80℃ for 2 h, hybridization was carried out in 10 ml DIG Easy Hyb (Roche) at 50℃ overnight. After stringency washes with 0.1×SSC/0.1% SDS at 68℃ for 2 times, the membrane was immunologically detected by using DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche).

Immunocytofluorescence

HEK293T Cells were seeded on sterile glass coverslips in a 6-well plate and incubated overnight to reach 60-80% confluent. Cells were then fixed with 4% paraformaldehyde for 15 min at room

temperature and washed with PBS 3 times. After permeabilizing with PBST for 10 min, cells were blocked with 10% goat serum in PBS for 1 h and incubated with the 7A9 mAb against human ANKIB1 [19] overnight at 4℃. The coverslips were washed with PBST 3 times followed by incubating with FITC-labeled goat anti-mouse IgG (Sigma-Aldrich) at 37℃ for 20 min. For nuclei staining, cells were incubated with DAPI (Amresco) diluted in PBST for 10 min at room temperature. Following the final washing with PBST, the coverslips were mounted onto slides using Glycerol Jelly Medium (Beyotime Biotech, China). Immunostained cells were imaged with a Nikon ECLIPSE 80i fluorescence microscope (Nikon, Japan).

Immunohistochemistry staining

SD rats were maintained by the Experimental Animal Center of Xinxiang Medical University. Brains of 3-day-old and adult SD rats were fixed with 4% paraformaldehyde, embedded in paraffin, and sectioned at 4 μm thickness. Immunohistochemical staining was performed as reference [21].7A9 mAb was used as a primary antibody.

Coimmunoprecipitation

HEK293T cells were incubated in 60 mm plates and transfected with 9 μg pCMV-Myc-ANKIB1 and 6 μg pFLAG-CMV-UbcH5B, pFLAG-CMV-UbcH6, pFLAG-CMV-UbcH7, pFLAG-CMV-UbcH8, pCMV-FLAG-UbcH10, or pCMV-FLAG-UbcH13. Coimmunoprecipitation assay was performed according to our previous study [22]. The anti-Myc mAb (Santa Cruz, sc-40), anti-FLAG mAb (Sigma-Aldrich, F3165) , and anti-ANKIB1 polyclonal antibody (Abcam, ab99346) were used for immunoprecipitation and immunoblotting.

In vitro ubiquitination assay

1 μg His_6-$ANKIB1^{RBR}$ or GST-ANKIB1(1-480) recombinant protein was incubated for 1 h at 30℃ in a 10 μL reaction mixture, consisted of 100 nM ubiquitin activating enzyme E1 (ENZO Life Sciences), 1 μM E2, 5 μM His_6-Ub, 50 mM Tris-HCl (pH 7.5), 2 mM ATP, 2.5 mM $MgCl_2$ and 1 mM DTT. The reaction was stopped by adding 10 μL 2×SDS loading buffer. The samples were boiled and separated on a 10% SDS-PAGE gel and immunoblotted with the anti-Ub polyclonal antibody (Abcam, ab7780).

In vivo ubiquitination assay

HEK293T cells were transiently transfected with pCMV-Myc-ANKIB1 and pRK5-HA-Ub expression plasmids. The cells were incubated for another 40 h in DMEM complete medium supplemented with 50 μM MG132 proteasome inhibitor (Sigma-Aldrich). Immunoprecipitation was then performed with an anti-Myc antibody. The precipitates were analyzed by immunoblotting with an anti-HA antibody (Abcam, ab9110).

Bioinformatic analysis of ANKIB1 protein

The structure of ANKIB1 protein was analyzed by Uniprot, PROSITE, InterPro, COILs Server, and SMART database. Multiple sequence alignment of ANKIB1 homologs from different species was performed using the ClustalX and was adjusted by hand.

Results

Structure and sequence analysis of ANKIB1

ANKIB1 can be divided into four parts from the amino-terminus to the carboxy-terminus: ANK domain, RBR supradomain, Ariadne domain, and the carboxy-terminal region (Fig. 1A). Like other human RBR proteins, ANKIB1 contains two C3HC4-type RING domains, RING1 and RING2. However, the IBR domain is C5HC2 type (also can be considered as C3HC2HC2 type) rather than C6HC in other human RBR proteins (Fig. 1B). The typical Ariadne domain is 150 to 165 AAs long with a sandwich structure of coiled-coil/leucine zipper/coiled-coil [23]. The leucine zipper in the middle is of mini type, with the consensus sequence of L-X(6)-L-X(6)-L. The Ariadne domain of ANKIB1 is structurally incomplete because it contains only one coiled-coil motif and a mini leucine zipper motif. However, there is another leucine zipper in the C-terminal region, which is overlapped with the UIM.

A comparison of the amino acid sequence of ANKIB1 homologs from different species revealed that the N-terminus of ANKIB1 is highly conserved, including the ANK domain, RBR supradomain, and the Ariadne domain (Fig.1B). The N-terminal 779 AAs of human ANKIB1 have 97.6%, 94.4%, 94.5% or 84.8% identity to that of mouse ANKIB1, emperor penguin ANKIB1, alligator mississippiensis ANKIB1, or zebrafish ANKIB1, respectively.

ANKIB1 has different expression patterns in human immortal cell lines

We have previously shown four ANKIB1 protein isoforms in the human brain, whose expression levels were 122 kDa, 88 kDa, 110 kDa, and 150 kDa from high to low [19]. The isoform of 122 kDa was coincident with Q9P2G1, the only acknowledged protein isoform deduced from the coding sequence of ANKIB1 transcript (NM_019004). In this study, we detected ANKIB1 protein in eight human cell lines by immunoblotting. Besides the 122 kDa and 88 kDa isoforms, a band of 66 kDa was detected in all cancer cells and IM-9 cells (Fig. 2A). All the cancer cell lines expressed the same three isoforms. Compared with the consistency of ANKIB1 expression in the four cancer cell lines, the three lymphocyte-derived cell lines showed amazing difference.

To evaluate the reliability of immunoblotting results, we further analyzed the transcriptional splicing variants of *ANKIB1* in the SH-SY5Y cells by Northern blotting. Three splice variants were exposed, which just corresponded to the three protein isoforms (Fig. 2B). We presume that these mRNA variants encode 122 kDa, 88 kDa, and 66 kDa ANKIB1 protein isoforms respectively.

ANKIB1 is mainly localized in the cytoplasm and highly expressed in the brain cortex

The endogenous ANKIB1 in HEK293T was detected by immunofluorescence. We observed intense fluorescence in the cytoplasm. In contrast, much less ANKIB1 was detected in the nuclei (Fig. 3A). Meanwhile, ANKIB1 was not uniformly distributed in the cytoplasm but presented a distinctive characteristic of local aggregation.

Next, we defined whether the two main ANKIB1 isoforms, 122 kDa and 88 kDa protein, expressed in the cytoplasm or the nucleus. The cytoplasmic or nuclear proteins were extracted from HEK293T cells and subjected to immunoblotting analysis. The 122 kDa ANKIB1 was the predominant isoform both in the whole cell and the cytoplasm, which is 3.5 or 6.2 times as much as the 88 kDa isoform. Conversely, the level of 88 kDa isoform was much higher than that of 122 kDa isoform in the nucleus (Fig. 3B).

We further defined the cell distribution of ANKIB1 in rat brain by immunohistochemical staining.

The analysis showed that there was a significant difference between the basal ganglia and the brain cortex. As shown in Fig. 4, ANKIB1 was widely and highly expressed in the brain cortex, but it was seldom detected in the basal ganglia, especially the nucleus accumbens.

ANKIB1 interacts with UbcH8, UbcH10, and UbcH13

A panel of class I E2s (UbcH5B, UbcH7, UbcH8, and UbcH13) and class III E2s (UbcH6 and UbcH10) expressing plasmids were co-transfected with pCMV-Myc-ANKIB1. The protein extracts of HEK293T cells containing FLAG-UbcHs and Myc-ANKIB1 were submitted to immunoprecipitation with anti-Myc antibody and the presence of FLAG-UbcHs in the immunoprecipitates were then detected by immunoblotting using anti-FLAG antibody (Fig. 5A). UbcH8, UbcH10, and UbcH13 were found to be coimmunoprecipitated with Myc-ANKIB1, but no detectable interaction was observed for UbcH5B, UbcH6, or UbcH7. Positive results were also obtained in testing the stable association between overexpressed UbcH8, UbcH10, or UbcH13 with endogenous ANKIB1 by reverse coimmunoprecipitation (Fig. 5B). These results indicate that ANKIB1 interacts with both class I and class III E2s.

ANKIB1 is an E3 ubiquitin ligase

The E3 activity of ANKIB1 was first determined in an *in vitro* model. Recombinant His_6-$ANKIB1^{RBR}$ that contained the minimal RBR supradomain of ANKIB1 was incubated with His_6-Ub in the presence of ATP, ubiquitin-activating enzyme E1, and UbcH8, which showed strong interaction with ANKIB1 and has been found to interact with several RBR ligases. UbcH7 has been reported to act as the common E2s for 12 characterized human RBR E3s except for ARA54 [24]. Contrary to UbcH7 and UbcH8, UbcH5s (including UbcH5A, UbcH5B, and UbcH5C) have not been found to interact with any RBR protein. So UbcH7 and UbcH5 were also set up to combine with His_6-$ANKIB1^{RBR}$ in the *in vitro* assay. Ubiquitin blotting detected protein smear in the UbcH8 system, while UbcH5 exhibited much weaker smear than UbcH8 did (Fig. 6A). Surprisingly, UbcH7 gave a positive result comparable to UbcH8. To further test whether RING2 is involved in ubiquitin transferring, GST-ANKIB1(1-480) which contains RING1 and IBR domain was also assessed by *in vitro* ubiquitination assay. No protein smear was seen in any $ANKIB1^{RB}$-UbcH combination (Fig. 6B). Thus the RING2 of ANKIB1 is necessary for ubiquitination.

To investigate if ANKIB1 is self-ubiquitinated in the cellular context, HEK239T cells were co-transfected with Myc-ANKIB1 and HA-Ub expression plasmids. Immunoprecipitation and immunoblotting analysis showed significant anti-HA immunoreactivity (Fig. 6C). It's evident that ANKIB1 ubiquitinates itself apart from assembles polyubiquitin, but its E3 activity *in vivo* is much stronger than *in vitro*. Taken together, these data indicate that ANKIB1 functions as a partner of an E3 ligase complex.

Discussion

ANKIB1 is the second-largest protein in the primary structure among all human RBR proteins. Like the largest RBR protein, CUL9, ANKIB1 is one of the only two RBR proteins with peculiar structures. ANKIB1 contains ankyrin-repeat and UIM, neither of which is found in other RBR proteins. ANKIB1 also contains a coiled-coil motif in its Ariadne domain. Similar to the ANK domain, coiled-coil is a ubiquitous protein motif that mediates protein-protein interactions among a wide range of proteins and

has been widely used in medical research [25]. Both ankyrin-repeat and coiled-coil domain can self-associate [6]. The ANK domain and coiled-coil motif provide convenient anchors or platforms for the homodimerisation of ANKIB1 itself or the interaction with other proteins. Indeed, we got a large number of cellular proteins that could specifically bind to ANKIB1 by using the anti-ANKIB1 mAb as a grasper (data not shown). ANKIB1 is also the only Ariadne protein without an acidic domain, but it contains an additional leucine zipper. The leucine zipper is a special type of coiled-coil motif and plays a more prominent role in mediating the formation of protein dimers. For this reason, it can be considered that the Ariadne domain and C-terminal region of ANKIB1 constitute a large variant Ariadne domain. Another important difference between ANKIB1 and other RBR proteins is that their IBR domains have different constitutions of cysteine and histidine. The IBR domain functions to cooperate with RING1 to interact with E2 ubiquitin-conjugating enzyme and transfer ubiquitin from E2~Ub conjugate to RING2 to form an E3~Ub covalent intermediate [9, 26]. Based on the significant structural differences between ANKIB1 and other RBR proteins, we speculate that ANKIB1 may have peculiar mechanisms in protein quality control and other processes.

Ariadne subfamily RBR proteins are different in the subcellular localization despite their similarities in structure. ARIH2 is predominantly localized in the nuclear, while CUL9 is localized in the cytoplasm [27, 28]. HHARI is the only Ariadne RBR that is distributed throughout the cell, with nuclear levels of expression considerably higher in all cell types except for SH-SY5Y cells [29]. Different from the above three Ariadne RBRs, our studies showed that ANKIB1 is both a cytoplasmic and a nuclear protein but predominantly expressed in the cytoplasm. ANKIB1 seems to be concentrated in some organelles. We have detected whether ANKIB1 is localized in the mitochondrion, but a negative result was obtained (data not shown). We expanded our studies on the expression of ANKIB1 in the rat brain and found that ANKIB1 was highly expressed in the brain cortex. We didn't identify in which kind of cells ANKIB1 mainly expressed. It is very likely that ANKIB1 is expressed both in neuron and glial cells because almost the entire brain cortex is ANKIB1-positive. In contrast, ANKIB1 is poorly expressed in the white matter, especially the nucleus accumbens.

Of the 14 human RBRs, only ANKIB1 and RNF217 have not been shown to act as E3 ligases or as a part of E3 complexes. To better understand the ubiquitin ligase function of the ANKIB1, we initially aimed to identify the cognate E2 conjugating enzymes. A panel of E2s has been proved to function with the human RBR ligases. The closely related class I E2s, UbcH7 and UbcH8, are known to act as the universal E2s for most human RBR E3s. UbcH7 has been found to interact with all well-studied RBR ligases except ARA54 [24]. Meanwhile, nine defined human RBR E3s have been shown to interact with UbcH8 except for ARIH2, but the interaction is weaker than that of UbcH7 for some RBRs[27, 30]. We screened a batch of E2s and found that ANKIB1 interacted with UbcH8, UbcH10, and UbcH13, but not with UbcH5B, UbcH6, and UbcH7. These results agree with the rule that human RBR proteins interact with at least one of the UbcH7 and UbcH8, and support the view that Ariadnes are the most complex and changeable RBR proteins.

It has been reported that the E3 ligase activity of some RBRs is autoinhibited [26, 31, 32]. In HHARI, the Ariadne domain has been shown to exert intramolecular autoinhibition by masking the RING2 active cysteine [33]. So we prepared truncated forms of ANKIB1 for testing E3 enzyme activity *in vitro*. Even so, His_6-$ANKIB1^{RBR}$ just showed weak E3 activity. Interestingly, UbcH7 and UbcH5B also support ANKIB1-dependent ubiquitination, although they do not interact with ANKIB1. Similar phenomena have been observed in other RBR proteins in the previous studies [9, 34]. One possibility is that there are very weak interactions between the E2s and E3s in the cell, but there are high

concentrations of E2s and E3s and few interference factors in the *in vitro* context, which is conducive to ubiquitination. Contrary to the *in vitro* ubiquitination assay, the over-expressed ANKIB1 is deeply auto-ubiquitinated *in vivo*. These results suggest that ANKIB1 is a catalytic part of an E3 ligase complex, and the low level of ubiquitination in the *in vitro* system is due to the lack of a (or some) cofactor(s) which can promote protein ubiquitination. Certainly, the UIM of ANKIB1 may also contribute to the enhancement of its E3 activity *in vivo*.

Most of the RBR proteins were first reported as independent E3s, but more and more evidence support that RBRs may function as a part of CRL complexes. CRLs comprise the largest class of multi-subunit E3 ligases. A canonical CRL consists of four components: a cullin protein, a catalytic RING protein, an adaptor protein, and a substrate receptor [35, 36]. Most RING proteins in the CRLs contain a RING-H2 domain, which is a variant type of the C3HC4 RING finger domain with the fourth cysteine residue displaced by a histidine. The IBR domain of ANKIB1 contains two conserved histidines, which is the same as the RING-H2 domain. It cannot be completely excluded that the IBR domain of ANKIB1 functions similarly to RING-H2 even though the order of cysteine and histidine are not the same in the two domains. As the most prevalent 'mobile' domain, the ANK domain often co-exists with F-box or SOCS box in proteins, which indicates that ANK is functionally related to F-box and SOCS-box. Meanwhile, more than one-third of the CRL substrate receptors belong to F-box or SOCS proteins [37]. These facts suggest that ANKIB1 may form a CRL complex with an F-box protein or a SOCS protein. Most importantly, Marín and Ferrús have hypothesized that Ariadne proteins may associate with cullin proteins to form ubiquitin ligase complexes, and it has been confirmed on Ariadne RBRs and Parkin [28, 38-41]. Based on these data, it is reasonable to propose that ANKIB1 may exert full E3 enzyme activity in the form of CRL in the cellular context. Future studies will be required to ascertain the components that comprise the CRL together with ANKIB1, and whether ANKIB1 can interact with different components to form different CRLs like Parkin.

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Figures

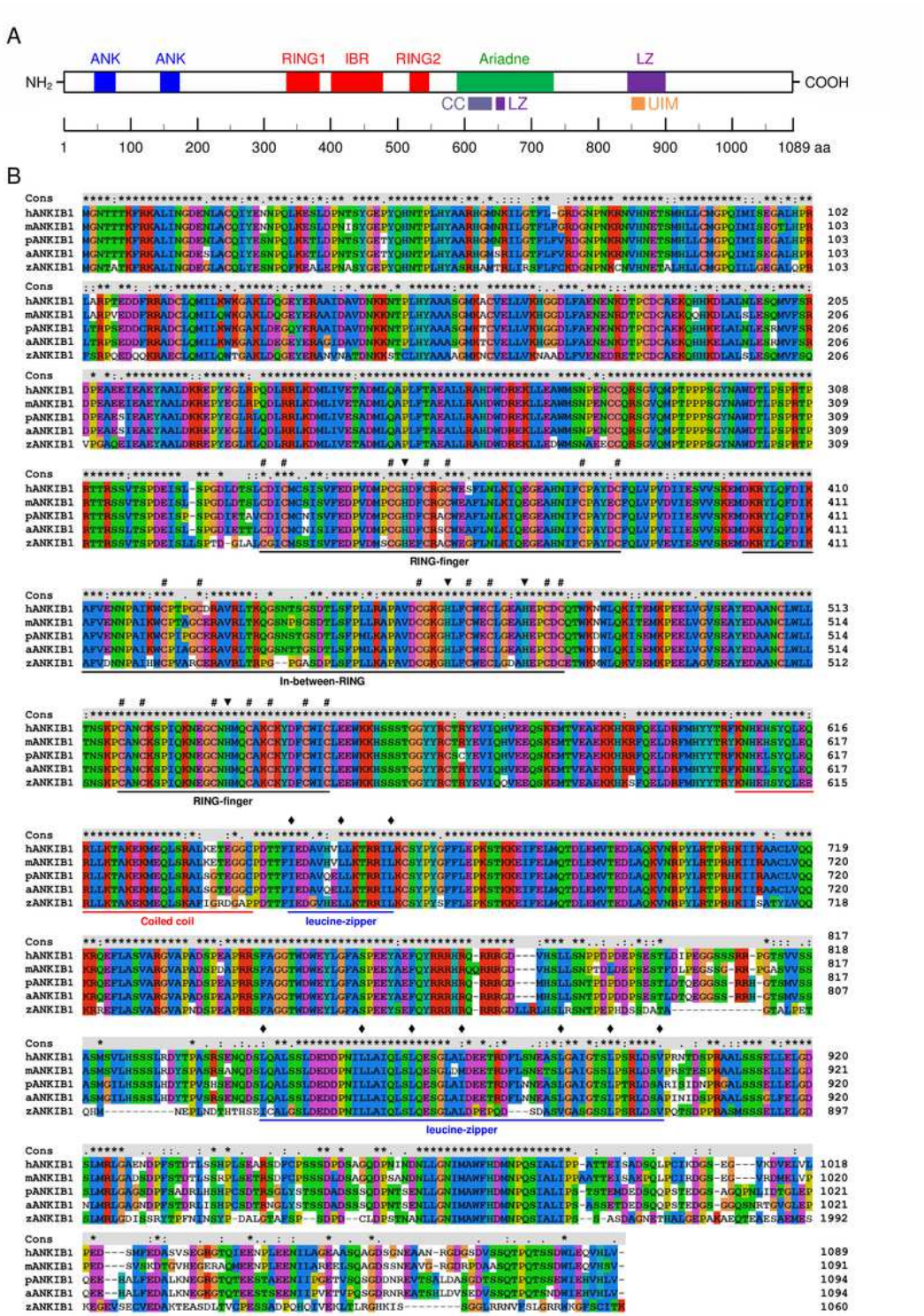

Figure 1

Structure of ANKIB1 protein. (a) Schematic representation of human ANKIB1 protein. The structure was predicted by SMART/Pfam with manual correction. CC, coiled-coil motif. LZ, leucine zipper motif. (b) Multiple sequence alignment of ANKIB1 orthologs from human (hANKIB1, Q9P2G1), mouse (mANKIB1,

NP_001276456), emperor penguin (pANKIB1, XP_009273943), alligator mississippiensis (aANKIB1, XP_006258998), and zebrafish (zANKIB1, NP_001074461). Numbers indicate ANKIB1 amino acid positions. Consensus amino acid residues were arranged in the top lines. Conservative cysteine (#) and histidine (□) residues are indicated in the RBR supradomain, and key residues of the leucine zipper are also labeled (□).

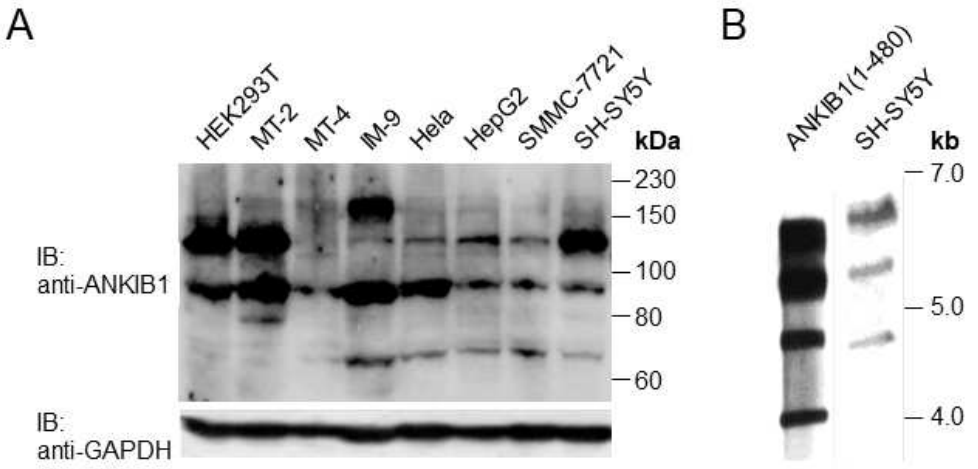

Figure 2

Analysis of the ANKIB1 protein isoforms and mRNA variants. (a) Immunoblotting analysis of ANKIB1 protein isoforms in human immortal cell lines. The whole cell lysate of each cell line containing 50 μg proteins was subjected to immunoblotting with the anti-ANKIB1 mAb as the primary antibody. (b) Northern blotting analysis of ANKIB1 mRNA variants in SH-SY5Y cells. ANKIB1-specific cDNA probe was synthesized by PCR and labeled with digoxigenin. Total RNA was isolated by guanidine isothiocyanate extraction from the SH-SY5Y cells and hybridized with ANKIB1 cDNA probe. Plasmid pCMV-Myc-ANKIB1(1-480) served as a positive control.

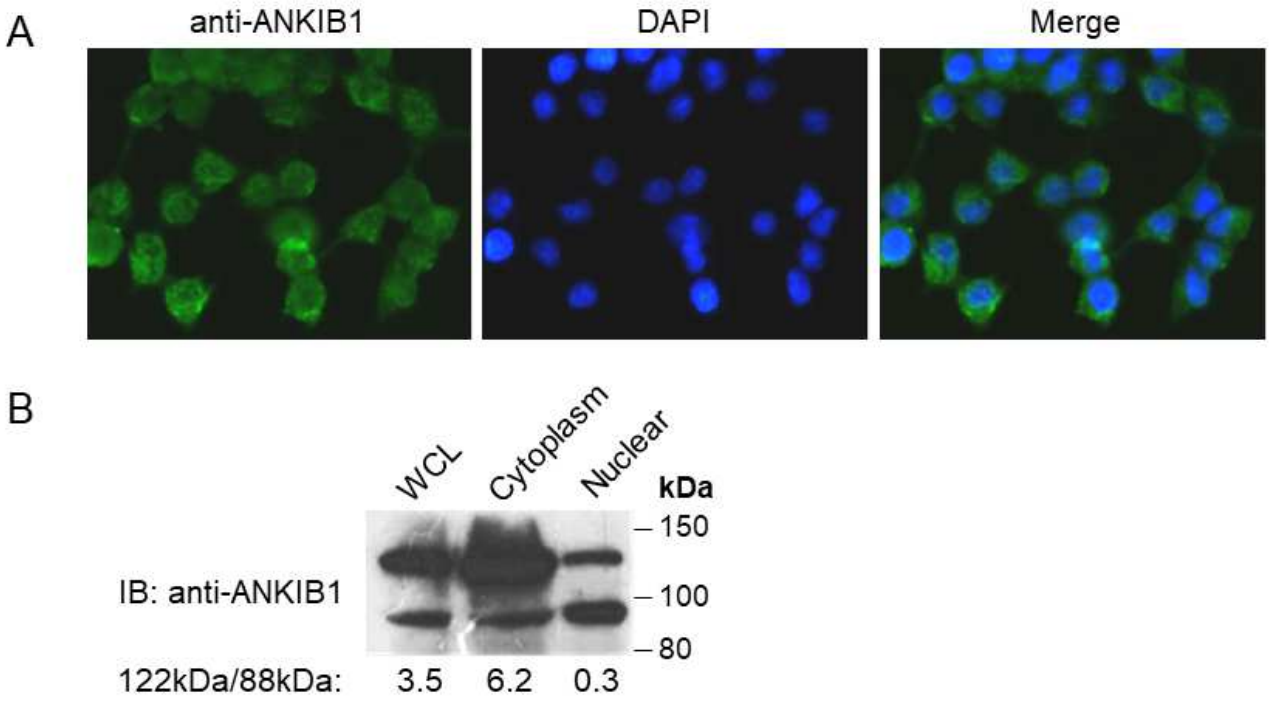

Figure 3

Localization of ANKIB1 in HEK293T cells. (a) Immunofluorescence staining of endogenous ANKIB1 in HEK293T cells. HEK293T cells were plated on glass coverslips. 24 h later, the cells were fixed and processed for immunofluorescence staining. Endogenous ANKIB1 was detected by using the anti-ANKIB1 mAb (green). The nucleus was visualized in blue using DAPI staining. (b) Distribution of ANKIB1 isoforms in the cytoplasm and nucleus of HEK293T cells. The cytoplasmic and nuclear proteins were extracted and subjected to immunoblotting with the anti-ANKIB1 mAb. The gray-scale ratios of the 122 kDa isoform to the 88 kDa isoform were calculated.

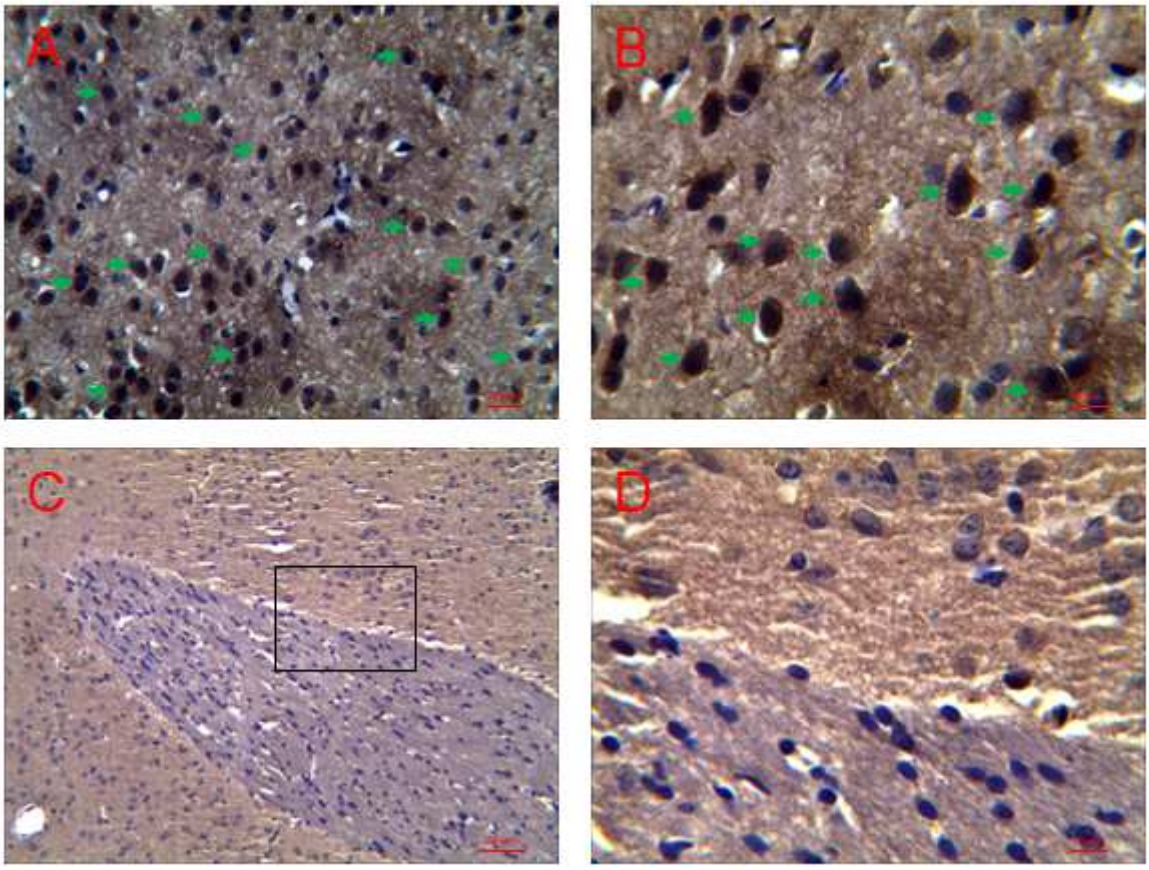

Figure 4

ANKIB1 expression detected by immunohistochemistry in rat brain cortex. (a) and (b) ANKIB1 expression in the brain cortex. (c) ANKIB1 expression in the basal ganglia. (d) Enlarged images of (c). Positive cells were defined as presenting buffy grains in cytoplasmas shown by arrows. Scalar bars present 20 μm in (a), 10 μm in (b) and (d), while 50 μm in (c).

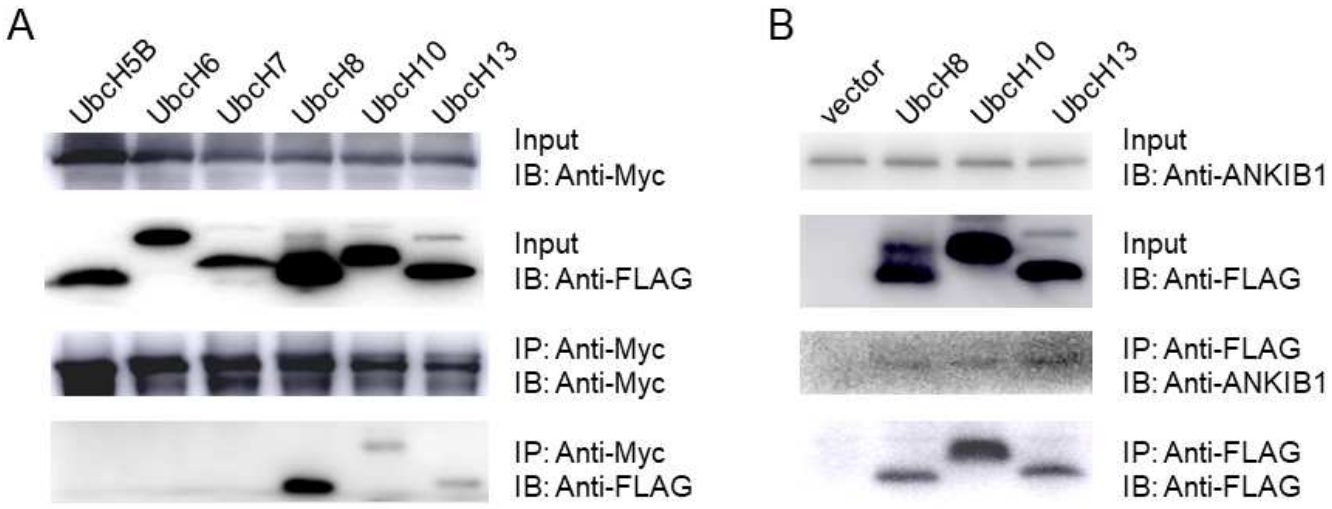

Figure 5

Association of ANKIB1 with UbcH8, UbcH10, and UbcH13. (a) Interaction between overexpressed ANKIB1 and E2s. 9 μg pCMV-Myc-ANKIB1 was co-transfected with 6 μg of every expression vector encoding FLAG-Ubc into HEK293T cells. Cells were lysed 48 h after transfection, and the lysates were subjected to immunoprecipitation with an anti-Myc antibody. The precipitates were resolved on SDS-PAGE gel and subjected to immunoblotting with anti-FLAG or anti-Myc antibody. (b) Interaction between endogenous ANKIB1 and overexpressed E2s. HEK293T cells were transfected with 6 μg of pFLAG-CMV-UbcH8, pCMV-FLAG-UbcH10, or pCMV-FLAG-UbcH13. Immunoprecipitates prepared by anti-FLAG antibody were subjected to immunoblotting with anti-FLAG mAb or anti-ANKIB1 polyclonal antibody.

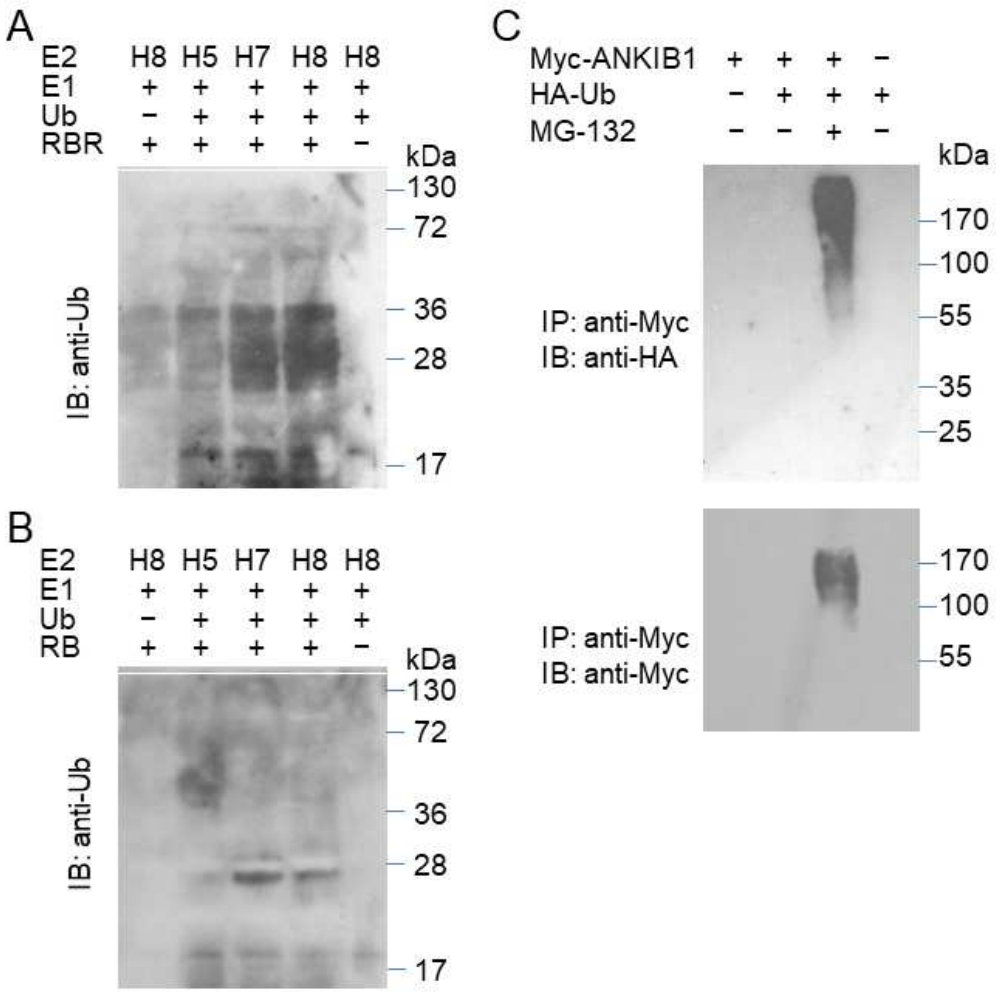

Figure 6

ANKIB1 is involved in E2-dependent self-ubiquitination both in vitro and in vivo. (a) In vitro ubiquitination assay. His6-ANKIB1RBR was incubated with recombinant His6-Ub, E1, and E2s at 30□ for 1 h. The products were resolved by SDS-PAGE and immunoblotted with an anti-Ub antibody. (b) In vitro ubiquitination assay. GST-ANKIB1(1-480) was incubated with recombinant His6-Ub, E1, and E2s at 30□ for 1 h. The products were resolved by SDS-PAGE and immunoblotted with an anti-Ub antibody. (c) In vivo ubiquitination assay. HEK293T cells were co-transfected with pCMV-Myc-ANKIB1 and pRK5-HA-Ub and incubated for 40 h in the presence of proteasome inhibitor, MG132. The lysates were subjected to immunoprecipitation with an anti-Myc antibody and immunoblotted with anti-HA or anti-Myc antibody.