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A shorter variant of BTBD2 as a novel negative regulator of IRF-associated signalling

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Abstract

IRF family members are important transcription factors involved in TLR and RLR signalling and type I IFN expression. Many cellular factors are reportedly associated with the IRF-mediated signalling pathways, but the IRF-associated signals are not completely understood yet. We identified BTBD2 as a novel cellular factor interacting with IRF-4 by the yeast two-hybrid system. A short BTBD2 variant transcript, termed variant 2 (v2), was found in various cell lines, expressed from an internal promoter in the third intron, in addition to the canonical BTBD2 transcript (v1). The canonical v1 protein did not bind to any IRFs tested, but the v2 protein bound to all the tested IRF family members. Furthermore, only the v2 protein inhibited TLR- and RLR-mediated signalling significantly, probably due to suppressing the nuclear translocation of IRFs. Knockdown of BTBD2 expression by siRNA enhanced TLR4-activated IFN-β and IL-12p40 expressions. Taken together, this study found that BTBD2 v2 negatively controls the IRF-associated signalling pathways, suggesting that BTBD2 v2 is one of the key regulatory factors involved in the negative feedback of IRF signalling.

Keywords: BTBD2; Interferon regulatory factors (IRFs); Toll-like Receptor 4 (TLR4).

INTRODUCTION

IFNs are cytokines that render cells resistant to viral infection and regulate cell growth and differentiation (Piehler *et al*., 2012). They elicit the pleiotropic biological effects mainly by regulating the expressions of many interferon stimulated genes (ISGs). Interferon regulatory factors (IRFs) were originally identified as regulators of IFN expression, but they also play important roles in inflammation by regulating expressions of the genes related to inflammation or by interacting with proteins that are involved in translation or ubiquitination (Tamura *et al*., 2008). In human, nine IRF members (IRF1~9) have been identified so far.

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IRF-4 is highly expressed in hematopoietic cells, and contains a DNA-binding domain (DBD) on the N-terminus, a transcription activation domain (AD) in the middle, and a repression domain (RpD) on the C-terminus (Eisenbeis *et al*., 1995). IRF-4 has been implicated in multiple myelomas (Yamada *et al*., 2001) and adult T cell leukemia and lymphoma (ATLL) (Imaizumi *et al*., 2001). To elucidate the activation mechanism, we had previously used the tandem affinity purification (TAP) method to isolate molecules including c-Rel, that bind to IRF-4 (Shindo *et al*., 2012), and here, we employed the yeast two-hybrid method to identify IRF-4 interacting proteins. Among them, the broad-complex, tramtrack, and bric-à-brac (BTB) domain containing 2 (BTBD2), double-strand-break repair protein rad21 homolog (RAD21 homolog), and myeloid/lymphoid or mixed-lineage leukemia 4 (MLL4) were isolated as IRF-4-binding proteins. We further analyzed the C-terminal fragment of BTBD2 (BTBD2-CT) isolated by the yeast two-hybrid, because the

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interaction between BTBD2-CT and IRF-4 was most evident. Surprisingly, besides IRF-4, the BTBD2-CT bound to IRF-2, IRF-3, IRF-5, and IRF-7, and inhibited their signal transductions.

To date, many molecules have been reported to modulate the IRF signalling. For example, IRF-4 cooperates with ETS protein to bind a composite DNA element containing an IRF-4-binding site adjacent of ETS binding motif (Eisenbeis *et al*., 1995). As a co-repressor, IRF-2-binding proteins (IRF-2BP1 and BP2) have been reported to bind to the C-terminal IRF-2 and co-repress transcription of several genes (Childs *et al*., 2003). In the case of IRF-3 and IRF-7, they form a large signalling complex with CREB-binding protein (CBP) or p300 in the nucleus, with phosphorylation by TBK or IKKε kinases, and subsequently translocates into the nucleus (Yoneyama *et al*., 1998). IRF-5 has been shown to interact with the RelA subunit of NF-kB, resulting in a high and sustained expression of TNF (Krausgruber *et al*., 2010). However, BTBD2-CT is unique in its binding to all the IRFs stated above. The canonical BTBD2 (variant 1) did not bind to any IRFs, but another short variant of BTBD2 (variant 2) transcribed from an internal promoter present in the third intron, bound to the IRFs and inhibited the IRF-associated signalling, similar to that observed with BTBD2-CT. In this study we characterized the BTBD2 v2 protein as a novel negative regulator of various IRF signalling.

MATERIALS AND METHODS

Yeast Two-hybrid System

The yeast two-hybrid system was used to identify the possible binding proteins to IRF-4. The bait contains the DNA-binding domain and a part of activation domain of IRF-4 in pGilda vector as described (Hayashi *et al*., 2009). Human T-cell mRNA was purchased from Ambion, and a random-primed cDNA library was constructed using a p424-GAL1-NLS-B42-AD vector. EGY48 yeast cells were transformed with the bait and the cDNA library sequentially. The clones were screened on the selection plates without leucine in the presence of X-Gal.

Cell culture

Human embryonic kidney (HEK) 293T, HeLa, Jurkat, KM3, THP-1 cells were used in this study. 293T and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Wako) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), at 37˚C in 5% CO_2. Jurkat, KM3 and THP-1 cells were maintained in RPMI1600 (Wako) with 10% FBS (Gibco), at 37 ˚C in 5% CO_2.

Plasmids

We amplified cDNA sequences of TLR3, TLR4, TRIF, MyD88, IRF-2, IRF-3, IRF-4, IRF-5, IRF-7, IKKε, MD2, BTBD2 v1, and BTBD2 v2 from HeLa or THP-1 cells by RT-PCR. The various deletion mutant constructs of BTBD2 were constructed by cutting at various restriction enzyme sites. All the clones generated by PCR were confirmed by DNA sequencing. The CD4-TLR4 expression plasmid was described previously (Yoshii *et al*., 2011). The promoter regions of IFNβ (-343 ~ +17), IL-12p40 (-340 ~ +26), and BTBD2 v2 (-438 ~ +36 relative to the transcript start site of UniProtKB/Swiss-Prot Q9BX70-2, Transcript ID ENST00000589685 as +1) were obtained from HeLa genomic DNA, and each of the them was inserted prior to the firefly luciferase reporter gene-containing pGL2-Basic vector (Promega). The five tandem kB luciferase reporter vector was purchased from Stratagene. The Op6-Luc containing six tandem repeats of operator sequence, and LexA-IRF-3 were described previously (Tokunaga *et al*., 2010), and the LexA-IRF-7 was also constructed by exchanging the DNA-binding domain with that of IRF-3.

Primers and siRNA

Sequences of the primers used in this study are listed in Table 1 *[Supplementary data]*. The BTBD2 siRNA and control GFP siRNA were purchased from Life Technologies Corporation and Greiner Bio-One Japan, respectively. Sequences of the sense strands are as follow: BTBD2 siRNA, 5'-GGAUUUGGGCUGUAUGGAUtt-3; GFP siRNA, 5'-GGAGUUGUCCCAAUUCUUGtt-3'.

Immunoprecipitation and Western Blot

HEK293T cells in 6-well plates were transfected with 0.5 µg pcDNA-FLAG-IRF expression plasmid together with or without 0.5µg pcDNA-HA-BTBD2 v1 or v2 expression plasmid, using FuGene 6 reagent (Roche Diagnostics). The transfected cells were lysed in 300µl of 1% NP40 lysis buffer, and 45 µl of the lysate was mixed with 4x SDS-sample buffer. The remaining lysate was precipitated with the anti-FLAG M2 antibody (Sigma) and protein A Sepharose 4B Fast Flow beads (Amersham Pharmacia Biotech). The lysates and immunoprecipitates were resolved by a gradient SDS-PAGE of increasing percentage, and immunoblotted with an anti-FLAG M2 antibody or anti-HA antibody (Covance) respectively.

Promoter activity assay

HEK293T in 24-well plates were transfected with 200 ng of the indicated firefly luciferase reporter plasmid using FuGene 6 transfection reagent. Luciferase

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activity was measured using Dual-Luciferase® Assay System (Promega), according to manufacturer's instructions.

RNA extraction, cDNA synthesis and quantitative PCR

HeLa, Jurkat, KM3 and THP-1 in 60mm dishes were harvested by RNeasy kit (Qiagen) according to manufacturer's protocol. cDNA was generated from 2 μg of RNA, using oligo dT primer and SuperScriptIII Reverse Transcriptase (Invitrogen). Quantitative real-time PCR was carried out using the indicated primers in Table 1 at their optimal conditions according to the manufacturer's protocol for the SYBR Green PCR kit (Qiagen).

RESULTS

A Novel Binding Protein to IRF Family Members

To identify the cellular factors interacting with IRF-4, a yeast two-hybrid screen was carried out using the N-terminal 1-200aa region of IRF-4 as bait, as described in Materials and Methods. The 7.9×10^6 library clones constructed from human T lymphocytes were screened on the selection plates without leucine in the presence of X-Gal, and six blue-coloured clones were isolated. Among them, three encode BTBD2, two encode double-strand-break repair protein rad21 homolog (RAD21 homolog), and one encodes myeloid/lymphoid or mixed-lineage leukaemia 4 (MLL4). All of the three BTBD2 clones contain the 204-467aa region (Fig. 1A, Fig. 1B). BTBD2, MLL4, and RAD21 homolog proteins were confirmed to interact with the 1-200aa fragment of IRF-4 weakly. However, only the C-terminal BTBD2 (BTBD2-CT) showed strong binding to the 136-277aa fragment containing the activator domain of IRF-4, but not to the 1-135aa fragment (Fig. 1A). These results indicate that the BTBD2-CT binds to the activation domain of IRF-4, and the same binding specificities were confirmed in mammalian cells when they were exogenously expressed in 293T cells (Data not shown). We further analyzed the property of BTBD2 in IRF signalling, because the BTBD2 binding to IRF-4 was most evident.

Two possible transcriptional variants (v1 and v2) of BTBD2

BTBD2 was previously cloned and characterized (Xu *et al*., 2002). In short, BTBD2, which is mapped to chromosome 19 (19p13.3→12), shares 80% similarity in amino acid sequence with BTBD1, a paralogous gene on chromosome 15 (15q24→26) (Carim-Todd *et al*., 2001). Proteins containing the BTB domain are currently classified into three categories: 1) DNA-binding proteins containing zinc finger domain in the C-terminus, 2) actin binding proteins which contain a Kelch repeat, 3) proteins with neither zinc finger domain nor Kelch repeat. Both BTBD1 and BTBD2 belong to the third category (Stogios *et al*., 2005). BTBD2 contains BTB and PHR (putative ubiquitin ligases PAM/*high-wire*/RPM-1) domains (Fig. 1B). The BTBD2-CT bound to the IRF-4 activation domain, which is also called IAD (IRF association domain), was shown to have some sequence homology among IRFs to form homo- or hetero-dimer between them (Tamura *et al*., 2008). To elucidate the function of endogenous BTBD2, we have cloned the full-length BTBD2 using cDNA obtained from HeLa cells. The BTBD2 genomic sequence contains nine exons (Fig. 2A), and the canonical mRNA is recorded as UniProtKB/Swiss-Prot Q9BX70-1, isoform1, Transcript ID ENST00000255608, encoding 525 amino acids. In addition, another shorter mRNA variant of BTBD2 containing six exons (the transcript starts in the third intron of the canonical variant) has been reported (UniProtKB/Swiss-Prot Q9BX70-2, isoform2, Transcript ID ENST00000589685, which encodes 204 amino acid that is the same as the C-terminal of the

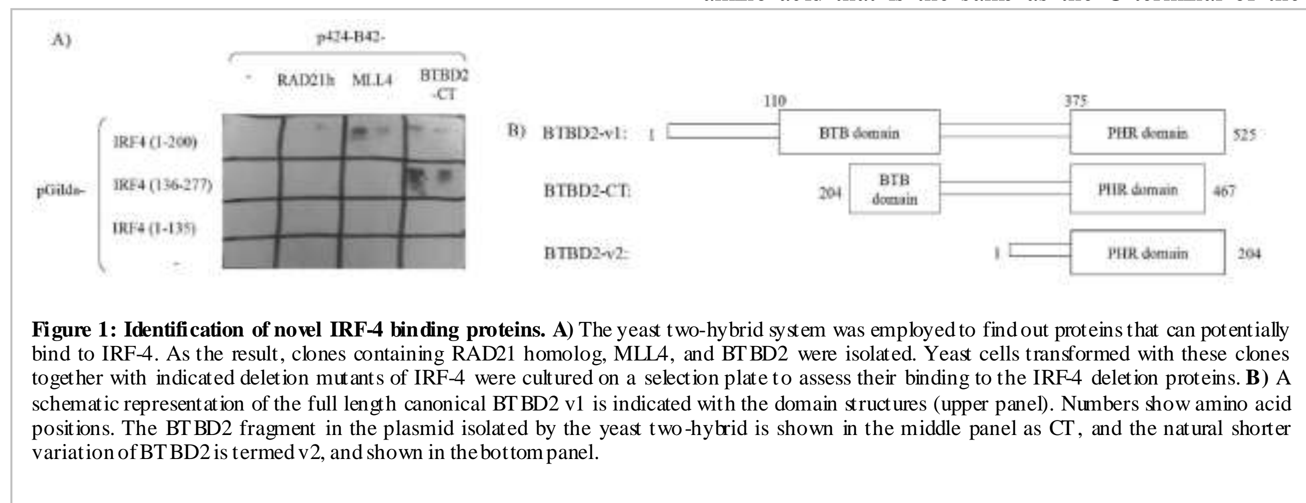

Figure 1: Identification of novel IRF-4 binding proteins. A) The yeast two-hybrid system was employed to find out proteins that can potentially bind to IRF-4. As the result, clones containing RAD21 homolog, MLL4, and BTBD2 were isolated. Yeast cells transformed with these clones together with indicated deletion mutants of IRF-4 were cultured on a selection plate to assess their binding to the IRF-4 deletion proteins. **B)** A schematic representation of the full length canonical BTBD2 v1 is indicated with the domain structures (upper panel). Numbers show amino acid positions. The BTBD2 fragment in the plasmid isolated by the yeast two-hybrid is shown in the middle panel as CT, and the natural shorter variation of BTBD2 is termed v2, and shown in the bottom panel.

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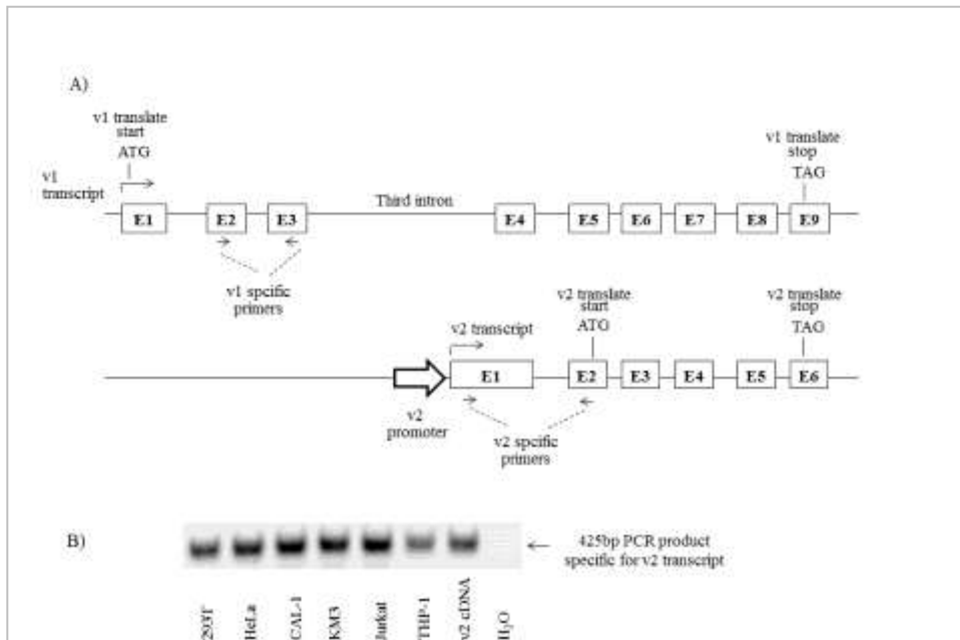

Figure 2: A BTBD2 shorter variant is expressed in various cell lines. A) BTBD2 genome structure is indicated. The full length canonical variant transcript (v1) has nine exons (E1~E9), while the shorter variant (v2) begins from the internal promoter in the third intron, and has six exons (E1~E6). The E5~E9 of v1 are the same as E2~E6 of v2. The v2 protein's translation is expected to start at Met^{1} and end at Thr^{204}, and encodes a 204 amino acid protein that is same as the same C-terminal region of v1 protein (Met^{322} to Thr^{525}). The v2 specific primers are indicated by arrows. **B)** The 425bp RT-PCR products amplified by the v2-specific primers from the indicated cell lines' mRNA, were subjected to agarose gel electrophoresis, and stained with ethidium bromide.

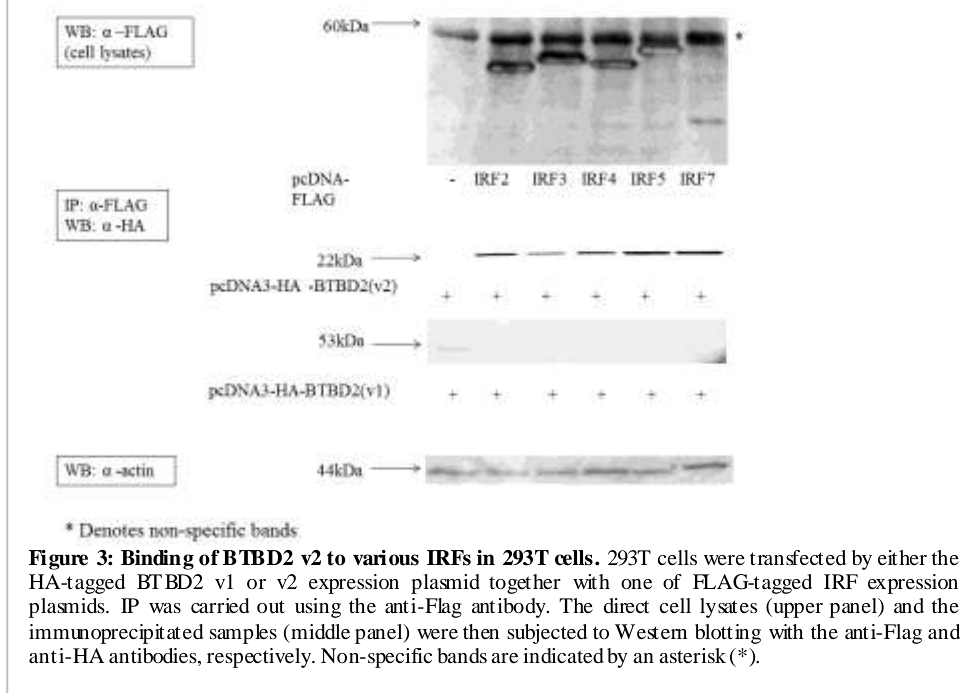

Figure 3: Binding of BTBD2 v2 to various IRFs in 293T cells. 293T cells were transfected by either the HA-tagged BTBD2 v1 or v2 expression plasmid together with one of FLAG-tagged IRF expression plasmids. IP was carried out using the anti-Flag antibody. The direct cell lysates (upper panel) and the immunoprecipitated samples (middle panel) were then subjected to Western blotting with the anti-Flag and anti-HA antibodies, respectively. Non-specific bands are indicated by an asterisk (*).

isoform1, starting at Met^{322} to Thr^{525}). To simplify the nomenclature, we called the isoform1 and isoform2 as v1 and v2, respectively.

Expression of BTBD2 short variant v2 in various cell lines

The BTBD2-CT lacks the N-terminal region containing BTB domain, like the putative v2 protein (Fig. 1B). Although the v2 transcript is registered in the UniProtKB/Swiss-Prot database, it is yet unknown how the shorter v2 mRNA variant is actually expressed endogenously, because the v2 transcript must begin in the third intron region of the BTBD2 gene. To detect the v2 transcript specifically by RT-PCR, without detecting the v1 transcript in the cDNAs and the possibly contaminating genomic DNA, we designed a pair of v2-specific PCR primers that amplify the 425bp fragment spanning from the third intron (v2 exon1, E1) to the v1 exon5, E5 (v2 exon2, E2) (Fig. 2A and Table 1). These primers do not amplify the v1 transcript because the third intron is excised in the v1 cDNA, but may amplify the possibly contaminating genomic DNA of 934bp. As shown in Fig. 2B, only the 425 bp fragment was found in a variety of cells tested, compared to the negative control (H_2O instead of cDNAs), showing that v2 is expressed in these cell lines. We cloned the 425 bp fragment from HeLa cell cDNA, and confirmed that the sequence was the same as UniProtKB/Swiss-Prot Q9BX70-2, isoform2, Transcript ID ENST00000589685. In addition, we cloned a 5'-region fragment spanning -874 ~ +32 relative to the v2 putative transcript start site in the Q9BX70-2 as +1 in the third intron, to examine whether the 0.9kb fragment has a promoter activity. The lysate prepared from the 293T cells which had been transfected for 24h with pGL2-Basic vector containing the 0.9kb fragment prior to the Luciferase gene, showed stronger Luciferase

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activity by more than ten times (13.75±2.29), when compared to the lysate from cells transfected with the empty vector. The 0.9 kb fragment contained many transcription factor binding sites, and a very high G/C-rich region (-520 ~ -360), with no canonical TATA box. Therefore, we conclude the v2 mRNA is expressed from this promoter region in these cell lines.

BTBD2 v2 binds to various members of the IRF family

To explore the v2 functions extensively, we first compared the binding specificities of v2 to IRFs with those of v1 using mammalian cells. The expression plasmids of HA-tagged BTBD2 v1 or v2, together with FLAG-tagged IRFs, were transfected into 293T cells. Co-IP was carried out using an anti-FLAG antibody, and subsequently Western blot (WB) was performed using an anti-HA antibody. Interestingly, BTBD2 v2 (23 kDa) was found to bind to all the IRF family members examined in the study, but BTBD2 v1 (53 kDa) could not interact with any of the IRF family members (Fig. 3). The expression of IRF-2, 3, 4, 5, and 7 in the cells were confirmed by immunoblotting of the cell lysates using the anti-FLAG antibody. Because the FLAG-tagged IRF-7 has a similar molecular size to a non-specific protein, the IRF7 protein was not distinctly detected, but the IRF-7-bound beads precipitated with BTBD2 v2. These results indicate that BTBD2 v2, but not v1, binds to the IRF-2, 3, 4, 5, and 7 proteins in the transfected 293T cells.

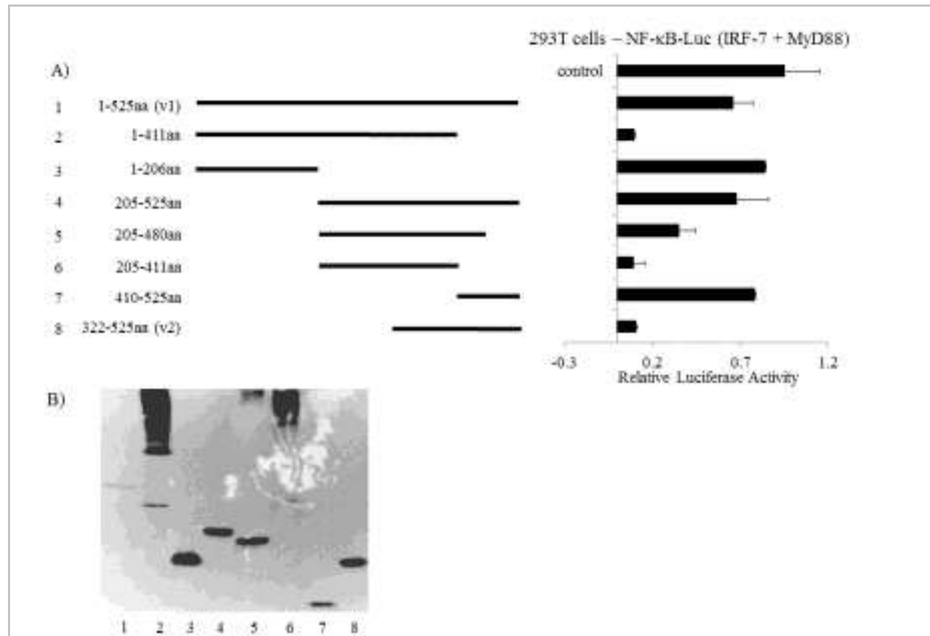

Figure 4: The C-terminal region of BTBD2 is essential for the suppressive activity on IRF-7 signal. A) The NF-κB-Luc reporter plasmid was transfected with the indicated BTBD2 deletion constructs in the presence of IRF-7 and MyD88 into 293T cells. Luciferase assay was carried out as NF-κB promoter activity 20h post-transfection. Relative values are indicated setting the intensities of cells without BTBD2 to 1. The experiments were done in duplicate, and the error bars show SD. **B)** Western blot of the transfected cells was performed using the anti-HA antibody.

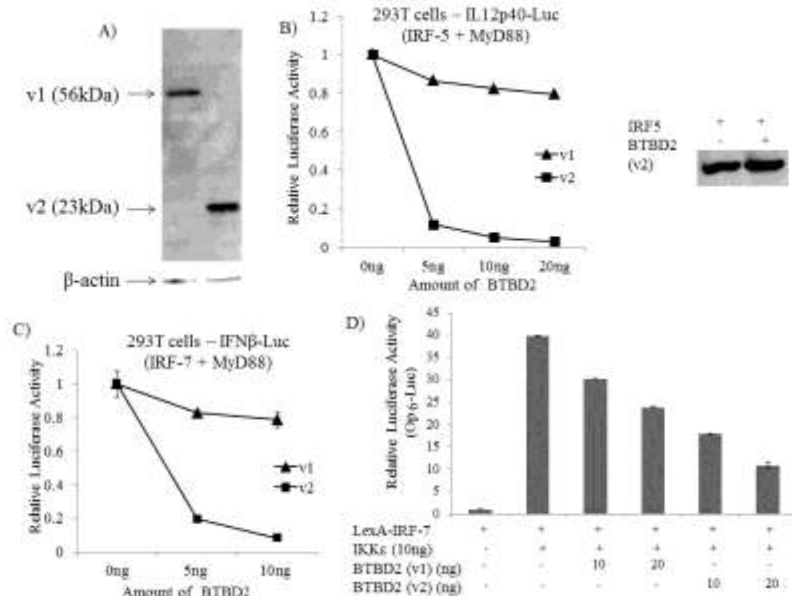

Figure 5: BTBD2 v2 suppresses the signalling mediated by IRF-5 and IRF-7. A) 293T cells were transfected by the HA-tagged BTBD2 v1 or v2 expression plasmid. Their expressions were confirmed by Western blotting using the anti-HA (upper panel) or anti-actin (lower panel) antibody. **B)** The IL12p40 promoter-driven Luc reporter plasmid was transfected with IRF-5 and MyD88 in the different amounts of BTBD2 v1 or v2 into 293T cells. The luciferase activities of transfected cells were measured as IL12p40 promoter activity (left). 293T cells were transfected with the FLAG-tagged IRF-5 expression plasmid with or without the BTBD2 v2 expression plasmid. Western blot of the cell lysates was carried out using the anti-FLAG antibody (right). **C)** Similarly, the IFNβ promoter activities of the transfected cells with or without the BTBD2 are as indicated. **D)** To measure the degree of nuclear translocation, the reporter Op6-Luc and LexA-IRF-7 plasmids were transfected into 293T cells together with the indicated plasmids. Luciferase assay was carried out 24h post-transfection. Relative values are indicated setting the intensities of cells without IKKε to 1. The experiments were done in triplicates, and the error bars show SD.

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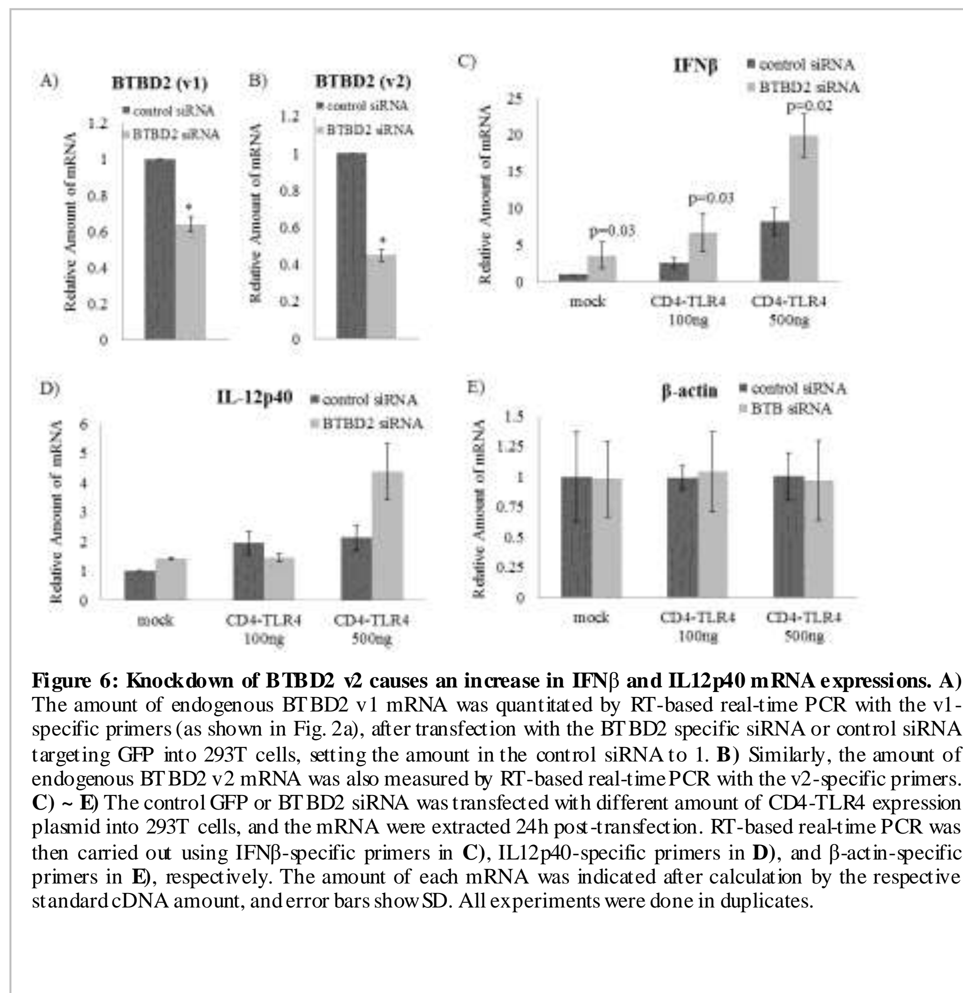

Figure 6: Knockdown of BTBD2 v2 causes an increase in IFNβ and IL12p40 mRNA expressions. A) The amount of endogenous BTBD2 v1 mRNA was quantitated by RT-based real-time PCR with the v1-specific primers (as shown in Fig. 2a), after transfection with the BTBD2 specific siRNA or control siRNA targeting GFP into 293T cells, setting the amount in the control siRNA to 1. **B)** Similarly, the amount of endogenous BTBD2 v2 mRNA was also measured by RT-based real-time PCR with the v2-specific primers. **C) ~ E)** The control GFP or BTBD2 siRNA was transfected with different amount of CD4-TLR4 expression plasmid into 293T cells, and the mRNA were extracted 24h post-transfection. RT-based real-time PCR was then carried out using IFNβ-specific primers in **C)**, IL12p40-specific primers in **D)**, and β-actin-specific primers in **E)**, respectively. The amount of each mRNA was indicated after calculation by the respective standard cDNA amount, and error bars show SD. All experiments were done in duplicates.

BTBD2 v2 suppresses various IRF signal pathways

BTBD2 v2 but not v1 inhibited the IRF-7- and MyD88-dependent NF-κB promoter activation (Fig. 4). To determine the site of BTBD2 responsible for inhibiting the IRF-dependent IFNβ and NF-κB promoter activation, we constructed a series of deletion mutants of BTBD2. The BTBD2 deletion mutants, 1-411aa, 205-480aa, and 205-411aa, suppressed the IRF-7-mediated activation of the NF-κB promoter, but the deletion mutants, 1-206aa, 205-525aa, and 410-525aa, did not. Taken together, it was suggested that the 205-411aa region of BTBD2 was responsible for the inhibition of IRF-7 signalling. The expression of the various deletion mutants of BTBD2 was confirmed in Fig. 4B.

The effects of BTBD2 v2 on IRF-5 were studied using similar luciferase reporter assays, using the IL-12 p40 promoter, because the activation of IL-12p40 promoter by TLR9 stimulation is known to be dependent on IRF-5 and MyD88 (Takaoka *et al*., 2005). Although expression levels of HA-tagged BTBD2 v1 and v2 proteins were similar (Fig. 5A), BTBD2 v2 suppressed the IRF-5 and MyD88-mediated activation of IL-12p40 promoter much more strongly than BTBD2 v1 (Fig. 5B). Because it had been suggested that BTBD2 has E3 ubiquitin ligase activity (Xu *et al*., 2003), BTBD2 v2 may suppress the signalling by degradation of IRF-5 protein. However, as shown in the right panel of Fig. 5B, BTBD2 v2 did not decrease the amount of IRF-5 protein. Similarly, BTBD2 v2 showed strong suppression of IRF-7 and MyD88-mediated activation of IFNβ promoter, but not BTBD2 v1 (Fig. 5C). These results indicate that BTBD2 v2 but not v1, significantly suppresses the IRF-mediated signal activation by an unknown mechanism other than degradation of IRF proteins.

We next examined the effects of BTBD2 on the IRF-3 and IRF-7 nuclear translocations by phosphorylation with TBK or IKKε kinase. The nuclear translocation of IRF-7 was assessed quantitatively by measuring luciferase activity with the translocation of LexA-IRF-7 fusion protein and subsequent activation of Op6-driven Luc gene, as described (Tokunaga *et al*., 2010). BTBD2 v2 suppressed significantly the nuclear translocation of IRF-7 in a dose-dependent manner. BTBD2 v1 also suppressed the nuclear translocation of IRF-7, but the effect of BTBD2 v1 was much weaker than that of v2 (Fig. 5D). These results support the notion that BTBD2 v2 inhibits the IKKε-mediated signal activation by binding to, and thereby preventing the nuclear translocation of IRF-7, because nuclear translocation is accompanied by the homo- or hetero-dimer formation between IRF-7 and IRF-3 (Wathelet *et al*., 1998).

If the inhibitory activities of BTBD2 v2 were due to binding and thus preventing IRFs-associated signalling, BTBD2 v2 is postulated to inhibit other TLR-activated IRF-mediated pathways. We have found that BTBD2 v2 inhibits TLR4-activated NF-κB promoter, and IFNβ promoter activations in 293T cells expressing pGL2-NF-κB-Luc, and pGL2-IFNb-Luc, respectively (Data not shown).

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Knockdown of BTBD2 enhances the up-regulation of IFNβ by TLR4 stimulation

To elucidate the effects of endogenous BTBD2 v2 on the regulation of IFNβ and NF-κB expressions by TLR4, we analysed the TLR4-mediated activation of IFNβ and IL-12p40 at the mRNA level after knocking down BTBD2 v2 with specific siRNA. We employed real-time PCR with the v2-specific primers used in Fig. 2D. As all the commercially available siRNA were designed to knockdown only BTBD2 v1, we used a siRNA targeting the common C-terminal region between v1 and v2. The siRNA actually knocked down the both mRNAs, as assessed by the real-time PCR (Figs. 6A and B). Total RNA was extracted from these cells, and IFNβ mRNA levels were quantitated by real-time PCR. The constitutively active mutant of TLR4 (Yoshii *et al.*, 2011), CD4-TLR4 elevated IFNβ mRNA expression in a dose-dependent manner (Fig. 6C). The amount of IFNβ mRNA transcript was significantly elevated by the BTBD2 knockdown. Also, IL-12p40 mRNA levels were elevated by CD4-TLR4 by about two-fold, and the BTBD2 siRNA further enhanced the expression of IL-12p40 mRNA (Fig. 6D). Levels of β-actin mRNA were not changed by the BTBD2 knockdown (Fig. 6E). These results indicate that the BTBD2 knockdown further activates TLR4-mediated induction of IFNβ and IL-12p40 expressions. These findings strongly support our hypothesis that the endogenous BTBD2 v2 negatively regulates TLR4 signalling.

DISCUSSION

Considering the importance of IRFs in various biological events, especially in immunological responses, the finding that BTBD2 v2 inhibited various IRF-dependent pathways is intriguing. The canonical BTBD2 v1 is thought to bind to topoisomerase I (TOPI) and TRIM5δ (an alternative splice variant of TRIM5α, a tripartite motif family member) in unique cytoplasmic bodies (Xu *et al.*, 2002, Xu *et al.*, 2003), but the BTBD2 v2 binds to various IRFs to prevent IFNβ and inflammatory cytokine productions. The tertiary structures of v1 and v2 are suggested to be different from each other. In v1, we speculate the N-terminal and C-terminal regions cover the middle IRF-binding and inhibitory domain. On the other hand, the IRF-binding region is likely to be exposed in v2, as the v2 protein lacks the N-terminal region of v1, and can therefore bind to IRFs, preventing interaction with various downstream molecules to transmit the signals. MyD88 is also reported to bind to several IRFs including IRF-1, IRF-4, IRF-5, and IRF-7, (Kawai et al., 2011). The hetero- or homo-dimerizations among IRF-3, IRF-5, and IRF-7 via their IAD1 regions, are essential for their nuclear translocation and transcriptional activation of effector genes (Cheng *et al.*, 2006, Marie *et al.*, 2000). MyD88 activates the IRF-dependent signalling, because of its association with TLRs or other molecules. In contrast to MyD88, the binding of BTBD2 v2 to IRFs inhibited the IRF functions.

BTBD2 v2 may also be a protease-dependent conversion of BTBD2 v1. In addition to BTBD2 v2 and BTBD2-CT, some deletion constructs either by removing N-terminal region or C-terminal region of BTBD2 v1, had inhibitory activities for the IRF-3-, and IRF-7-dependent IFNβ promoter activations, as shown in Fig. 4. Some virus proteases play important roles for their entry and proliferation in the infected cells (Fun *et al.*, 2012), or sometimes viruses employ cellular proteases for their infection (Humilton *et al.*, 2012). BTBD2 v1 may be a target of viral proteases for their efficient infection and proliferation. If some virus-activated cellular proteases or viral proteases themselves convert BTBD2 v1 to inhibitory forms of IRF signals, it is preferable for virus to proliferate.

Although we have shown the BTBD2 v2 transcript exists in many cell lines, we could not detect the BTBD2 protein expressions in those cells, because available antibodies to BTBD2 were poor to detect not only BTBD2 v2 but also the canonical BTBD2 v1 protein so far.

Finally, the BTBD2 promoter activation by the activated TLR4 can be a negative feedback mechanism to prevent excess signals for homeostasis. Excessive cytokine productions by microbial infections or environmental stresses sometimes cause adverse effects to the host, such as development of autoimmune disorder and tissue damage.

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Conflict of interest

The authors declare no conflict of interest.

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