**Drosophila** TAB2 is required for the immune activation of JNK and NF-kappaB

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**Abstract**

The TAK1 plays a pivotal role in the innate immune response of *Drosophila* by controlling the activation of JNK and NF-kappaB. Activation of TAK1 in mammals is mediated by two TAK1-binding proteins, TAB1 and TAB2, but the role of the TAB proteins in the immune response of *Drosophila* has not yet been established. Here, we report the identification of a TAB2-like protein in *Drosophila* called dTAB2. dTAB2 can interact with dTAK1, and stimulate the activation of the JNK and NF-kB signaling pathway. Furthermore, we have found that silencing of dTAB2 expression by dsRNAi inhibits JNK activation by peptidoglycans (PGN), but not by NaCl or sorbitol. In addition, suppression of dTAB2 blocked PGN-induced expression of antibacterial peptide genes, a function normally mediated by the activation of NF-kappaB signaling pathway. No significant effect on p38 activation by dTAB2 was found. These results suggest that dTAB2 is specifically required for PGN-induced activation of JNK and NF-kappaB signaling pathways.

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1. Introduction

The innate immune system of *Drosophila* responds to microbial infection by rapidly and massively producing antimicrobial peptides [1–4]. The induction of antimicrobial peptide gene is mediated by two distinct signaling pathways, the IMD and the Toll cascades [5,6]. The IMD pathway is usually activated by Gram-negative bacterial pathogens and leads to the production of antibacterial peptides, such as Attacin, Diptericin, and Cecropin, whereas the Toll pathway recognizes Gram-positive bacteria and fungal pathogens, driving the production of antifungal peptides such as Drosomycin [7–9]. Both signaling cascades require the activation of NF-kappaB to induce the transcription of antimicrobial peptide genes [10]. *Drosophila* TAK1, a member of MAPK kinase kinase, is essential for the activation of NF-kappaB by Gram-negative PGN, a functional component in commercial lipopolysaccharides (LPS) from Gram-negative bacteria [11,12]. Silencing of dTAK1 by RNAi abolishes PGN-induced I KK and JNK activation, and the production of antimicrobial peptides in *Drosophila* Schneider (S2) cells [13,14]. Furthermore, a *Drosophila* TAK1 mutant is defective in the production of antimicrobial peptides and highly susceptible to the Gram-negative bacterial infection [15].

The TGF-β (transforming growth factor-activated protein kinase (TAK1)) was originally identified as a TGF-β-activated MAPK kinase kinase, and was subsequently shown to play an important role in intracellular responses to pro-inflammatory
cytokines, such as interleukin-1 (IL-1) and tumor necrosis factor (TNF) [16–18]. In mammalian cells, activation of TAK1 requires an upstream kinase complex consisting of TAK1 and two specific TAK1-binding proteins, TAB1 and TAB2. TAB1 functions as an activator of TAK1, and TAB2 functions as an adaptor protein that links TAK1 to the upstream regulators TRAF6 in the IL-1 signaling pathway [19–21]. TAB2 localizes within membrane fraction of unstimulated cells. Upon stimulation by IL-1, TAB2 translocates from membrane to the cytosol and facilitates the interaction between TAK1 and TRAF6 [22,23]. A biochemical study has shown that TRAF6 is a RING domain-containing ubiquitin ligase that associates with a [22,23]. TAB2 translocates from membrane to the cytosol and facilitates the interaction between TAK1 and TRAF6 [22,23]. A biochemical study has shown that TRAF6 is a RING domain-containing ubiquitin ligase that associates with a [22,23]. Binding of TAB2/3 to poly-ubiquitinated TRAF2 or TRAF6 leads to the activation of TAK1 and IKK in a proteasome-dependent manner [21,25,26].

In mammals, the TAB2 deficiency is embryonic lethal due to liver degeneration and apoptosis, which is similar to that of NF-kappaB, IKK-beta-, and NEMO/IKK-deficient phenotypes [27]. However, activation of NF-kappaB and JNK MAP kinase by IL-1 and TNF appears to be normal in TAB2-deficient embryonic fibroblasts [27]. This phenotype was recently explained by the identification of another TAB2 homologue, TAB3 [25,28,29]. Indeed, simultaneous suppression of both TAB2 and TAB3 by RNAi inhibit the activation of IKK and JNK by IL-1 and TNF, suggesting that TAB2 and TAB3 function redundantly as mediators of TAK1 activation in IL-1 and TNF signaling pathways [25].

Compared to the study of TAB2 in mammalian cells, no functional role of TAB2 in the activation of JNK MAP kinase and NF-kappaB signaling pathway in Drosophila has been reported. In this study, we have demonstrated that dTAB2 binds to dTAK1, and overexpression of dTAB2 leads to the activation of NF-kappaB signaling pathway. Furthermore, suppression of the expression of dTAK2 by RNAi significantly reduced PGN-induced JNK activation and expression of antimicrobial peptides, indicating that TAB2 is involved in the activation of JNK and NF-kappaB in innate immune responses.

2. Experimental procedures

2.1. Molecular cloning

Full-length cDNA encoding dTAB2 was amplified from the total mRNAs of Drosophila S2* cells by using RT-PCR. The dTAB2 gene was then inserted into the pCDNA3.0 plasmid that contains a FLAG tag in its N-terminal. Full-length cDNA encoding dTAK1 was kindly provided by Jin Mo Park (University of California, San Diego). The cDNA of dTAK1 was then subcloned to pCDNA3.0 plasmid containing a HA tag at its N-terminal.

2.2. Cell culture and transfection—Drosophila

S2* cells were cultured in 1× Schneider's Drosophila media (GIBCO) supplemented with 10% FBS (GIBCO), 50 units/ml penicillin, and 50 μg/ml streptomycin at 25 °C [30]. For PGN stimulation, S2* cells were incubated with 1 μM 20-hydroxyecdysone (Sigma) to induce differentiation for at least 24 h prior to the addition of PGN [31,32]. HEK-293T cells were grown in MEM (Sigma) supplemented with 10% FBS (GIBCO), 1 mM l-glutamine, and 100 units/ml of penicillin/streptomycin at 37 °C under 10% CO2. HEK 293T cells were transfected using calcium phosphate method.

2.3. RT-PCR and dsRNA production

Total RNA was isolated from S2* cells using the Trizol Reagent (Invitrogen). First-strand cDNA was synthesized from total RNA by using the Superscript™ First-strand Synthesis System for RT-PCR (Invitrogen). The primer sequences used to generate dTAB2 dsRNAs are as follows and include a 5′ T7 RNA polymerase-binding site (TAATACGACTCACTATAGG-GGAG): forward, 5′ GTTGGTAGCCACACTCTGG3′; reverse 5′ GCCGCGACTGAGAACACT 3′. These primers were also used for knock-down efficiency detection. The following PCR program was used: an initial denaturation at 94 °C for 3 min followed by 30 cycles of amplification (95 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min) and an additional 10 min at 72 °C. The purified PCR products were used as templates to produce dsRNA using a MEGASCRIPT T7 transcription kit (Ambion). The dsRNA products were then precipitated with LiCl and resuspended in DEPC-H2O. dsRNA was annealed at 65 °C for 30 min followed by slow cooling to room temperature. RNA concentration was measured at A260 and in 1% agarose gels. dsRNAs were stored at −70 °C.

2.4. RNAi in S2* cells

RNAi was conducted according to the protocol described before [32,33]. Briefly, 1 × 10^6 S2* cells were plated in 1 ml of 1× Schneider's Drosophila media (GIBCO) without FBS per well of 6-well culture dishes. dsRNA was added at room temperature for 1 h, followed by 2 ml of 1× Schneider's Drosophila media (GIBCO) with 15% FBS. The cells were incubated for an additional 72 h to allow for turnover of the target mRNA.

2.5. Western blotting and coimmunoprecipitation

After 3 days of incubation with dsRNAs, S2* cells exposed to various stress conditions were lysed in SDS-PAGE sample buffer. The same amount of proteins was resolved by SDS-PAGE (10% gels) and transferred to nitrocellulose membranes. Antibodies that recognize phosphorylated p38 (Bio-source), phosphorylated JNK (Cell Signaling Technology) and γ-tubulin (Sigma) were used for Western blotting. For coimmunoprecipitation, HEK-293T cell lysates were prepared using radioimmunoprecipitation assay buffer (150 mM NaCl, 20 mM Tris–Cl pH 7.5, 1 mM glycerophosphate, 1 mM EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 0.1% Triton X-100, 1 mM Na3VO4, 1 μg/ml protease-inhibitor) and immunoprecipitated with M2 anti-FLAG antibody conjugated
to agarose beads and anti-HA agarose beads, respectively (Sigma), and the elute was separated by SDS-PAGE, transferred to a nitrocellulose membranes, and immunoblotted with anti-HA and anti-FLAG antibody (Sigma), respectively.

2.6. Real-time quantitative RT-PCR

Total RNA was isolated from S2* cells by using Trizol reagent (GIBCO), and then dissolved in 20 μl of RNase-free water. Followed by DNase (Promega) treatment, total RNA (1 μg) was used in 20 μl of reverse transcription reaction by using ReverTraAce reverse transcriptase (Toyobo) and oligo(dT)15 primer (Promega). The first-strand cDNA was used as a template for the quantitative RT-PCR. Expression of Attacin A and Diptericin was determined by real-time PCR using SYBR Green Master Mix (Toyobo). Thermocycler conditions included an initial holding at 50 °C for 2 min, then 95 °C for 10 min; this was followed by a 2-step PCR program: 95 °C for 15 s and 60 °C for 60 s for 40 cycles. Data were collected and quantitatively analyzed on an ABI PRISM 7900 sequence detection system (Applied Biosystems). PCR specificity was confirmed by the molecular weight of the PCR products and melting curve analysis at each data point. The copy numbers of attacin A and Diptericin were standardized against that of rp49 in each sample. Primers were as follows: Diptericin, 5′-GTTCACCATTGCCGTCGCCTTAC-3′, 5′-CCCAAGTGCTGTCCATTCCCTCC-3′; Attacin, 5′-GTGGTGGGTCAGGTTTTCGC-3′, 5′-TGTCCGTTGATGTGGGAGTA-3′; Rp49, 5′-AGATCGTAGAAGAAGCGCACCAAG-3′, 5′-CACCAGGAACCTTCTTGAAATCCGG-3′.

2.7. Luciferase assay

HEK 293T cells were cotransfected with pNF-kB-TA-Luc, a NF-κB reporter plasmid, pRL-TK (Clontech), and other plasmids. After 48 h transfection, cells were lysed for 15 min at room temperature (Passive Lysis Buffer, Promega). The lysate was analyzed using the Dual-Luciferase Reporter Assay System (Promega) and measured in a luminometer (Lumat LB 9507, Germany). Results are presented as the average of three measurements.

3. Results

3.1. Identification of dTAB2

In order to identify the TAB2-like protein in Drosophila, we searched the Drosophila genome database for proteins related to the human TAB2. A TAB2-related protein, namely dTAB2 was identified in Drosophila genome, which corresponds to locus CG7417 in FlyBase, known also as ORF1. We next designed the primers and amplified the cDNA encoding dTAB2 from the total RNA prepared from Drosophila S2 cells by RT-PCR (Fig. 1A). The dTAB2 gene was fully sequenced and assigned a GeneBank accession number DQ025530. The dTAB2 was predicted to consist of 831 amino acid residues. Similar to mammalian TAB2/3, dTAB2 contains an ubiquitin-binding domain (N-CUE) at its N-terminal and a highly conserved Zinc Finger (ZnF) domain at its C-terminal, respectively (Fig. 1B).

3.2. Association of dTAB2 with dTAK1 in vivo

Previous studies have shown that the mammalian TAB2/3 can interact with TAK1 through its C-terminal region, and the TAK1–TAB2–TRAF6 complex is important for IL-1 induced JNK and NF-κB activation in mammalian cells [20,25,26]. We next examined whether Drosophila TAB2 is also associated with dTAK1. HEK-293 cells were transfected with plasmids encoding HA-dTAK1 and FLAG-dTAB2, and dTAB2 was immunoprecipitated from cell lysates using anti-FLAG antibody (M2) conjugated to agarose beads. As shown in Fig. 2A, dTAB2 was detected in dTAK1 immunoprecipitates (Fig. 2B). These results indicate that dTAB2 binds with dTAK1 in vivo.

3.3. Activation of JNK and NF-kappaB signaling pathway by TAB2

It has been shown that association of mammalian TAB2/3 with TAK1 leads to the activation of JNK and NF-kB signaling pathway [13,14,20,25,26]. Since we have found that dTAB2 is
associated with dTAK1, we then investigated whether dTAB2 can also induce the activation of TAK1 and JNK activation. As indicated in Fig. 3A and B, overexpression of dTAB2 induced TAK1's activity which is indicated by enhanced phosphorylation of MKK6, and the activation of JNK.

Also, overexpression of dTAB2 induced the phosphorylation and degradation of IkB (Fig. 4A). Furthermore, when dTAB2 and an NF-kB-dependent luciferase reporter were cotransfected into 293 cells, dTAB2 was found to activate the reporter gene in a dose-dependent manner (Fig. 4B and C). However, deletion of C-terminal ZnF domain, but not of N-terminal CUE eliminated the activity of dTAB2 to induce NF-kappaB activation. These results suggest that only C-terminal ZnF region is required for the function of dTAB2.

### 3.4. Requirement of dTAB2 for PGN, but not sorbitol or NaCl-induced JNK activation

*Drosophila* TAK1 has been shown to be essential for the activation of JNK signaling pathway when stimulated with PGN [13–15]. To evaluate whether dTAB2 is also involved in PGN-induced JNK activation, the expression of dTAB2 was suppressed in Drosophila S2* cells using RNAi. Addition of dsRNA targeting dTAB2 eliminated their expression as determined by RT-PCR, whereas expression of RP49 was not affected (Fig. 5A). S2* cells treated or not treated with the dsRNA of dTAB2 were exposed to commercial LPS. It has been clearly shown that LPS does not activate the Drosophila IMD pathway. Instead, the active component, which is found in commercial LPS samples, is Gram-negative peptidoglycan (PGN) [11,12]. The cell lysates were subjected to Western blot analysis, and the activation of JNK was detected by using anti-phospho-JNK antibody. As shown in Fig. 5B, PGN-induced activation of JNK was significantly reduced when the expression of dTAB2 is abolished, suggesting that dTAB2 is essential for activation of JNK signaling pathway in response to PGN stimulation.

**Fig. 2. Interaction of dTAB2 with dTAK1.** The 293T cells were transfected with plasmid encoding HA-dTAK1, FLAG-dTAB2 as indicated. (A) The complexes were immunoprecipitated with M2 (anti-FLAG) agarose beads, and then immunoblotted with anti-HA or anti-FLAG antibodies. Whole-cell extracts were immunoblotted with anti-HA antibody. The complexes were immunoprecipitated with anti-HA agarose beads, and then immunoblotted with anti-FLAG or anti-HA antibodies. Whole-cell extracts were immunoblotted with anti-FLAG. Experiments were performed three times with comparable results.

**Fig. 3. Activation of JNK signaling pathway by dTAB2.** (A) HEK 293T cells cultured in 6-well plates were transfected with 2 μg PCDNA3.0 plasmid and PCDNA3.0–dTAB2, respectively. After 24 h, cell lysates were subjected to Western blot using anti-IκB, anti-p-IκB (Cell Signaling Technology) and anti-actin (Sigma). (B) HEK 293 cells cultured in 12-well plates were cotransfected with 0.3 μg β-gal or 0.15, 0.3 μg dTAB2, 0.3 μg dTAB2ΔN, 0.3 μg dTAB2ΔC with 0.1 μg pNF-kb-TA-Luc, and 0.1 μg pRL-TK. After 48 h, cells were lysed and luciferase activity was measured using Dual-Luciferase Reporter Assay System. Protein expression of dTAB2, dTAB2ΔN and dTAB2ΔC was detected by using anti-FLAG antibody (C). The data presented were mean±S.D. of 4 independent experiments.

**Fig. 4. Activation of NF-κB signaling pathway by dTAB2.** (A) HEK 293T cells cultured in 6-well plates were transfected with 2 μg PCDNA3.0 plasmid and PCDNA3.0–dTAB2, respectively. After 24 h, cell lysates were subjected to Western blot using anti-IκB, anti-p-IκB (Cell Signaling Technology) and anti-actin (Sigma). (B) HEK 293 cells cultured in 12-well plates were cotransfected with 0.3 μg β-gal or 0.15, 0.3 μg dTAB2, 0.3 μg dTAB2ΔN, 0.3 μg dTAB2ΔC with 0.1 μg pNF-kb-TA-Luc, and 0.1 μg pRL-TK. After 48 h, cells were lysed and luciferase activity was measured using Dual-Luciferase Reporter Assay System. Protein expression of dTAB2, dTAB2ΔN and dTAB2ΔC was detected by using anti-FLAG antibody (C). The data presented were mean±S.D. of 4 independent experiments.
have also checked the activation of p38 in the same condition, but found that PGN-induced p38 activation is not significantly affected by the abolishing of dTAB2 (Fig. 5B). This result is consistent with our previous findings that dTAK1 is not involved in the p38 activation in response to PGN stimulation [32].

We have also investigated whether dTAB2 is involved in the sorbitol or NaCl-induced JNK activation. Activation of JNK by treatment of sorbitol or NaCl is not significantly affected by the silencing of dTAB2 in Drosophila cells (Fig. 5C and D).

3.5. Requirement of dTAB2 for PGN-induced expression of antibacterial peptide gene

Upon stimulation with microbial pathogens, Drosophila cells respond to microbial infection by producing antimicrobial peptides. We have investigated the role of dTAB2 in the expression of these peptides in response to PGN stimulation.

Fig. 5. Requirement of dTAB2 for PGN-induced, but not sorbitol or NaCl-induced JNK activation. (A) Silencing of dTAB2 in Drosophila S2* cells by RNAi. S2* cells were untreated or treated with dTAB2-specific dsRNAs for 3 days. Expression of dTAB2 was determined by RT-PCR using specific primers as described above. Data shown are representative of three independent experiments. S2* cells treated with or without dTAB2 dsRNA were exposed to the stimulation of PGN (B), sorbitol (C), NaCl (D), respectively for 30 min. Cell lysates were then subjected to Western blot analysis using anti-phospho-JNK and anti-phospho-p38. Western blot analysis for γ-tubulin indicates equivalent amounts of protein present in each lane. Data shown are representative of 4 independent experiments.

Fig. 6. Requirement of dTAB2 for the up-regulation of anti-microorganism peptide mRNA. S2* cells treated with or without dsRNA specific for dTAB2 were exposed to PGN for the indicated times. Total RNA was extracted and subjected to real-time RT-PCR by primers specific for Diptericin (A) and Attacin (B). The copy number of Diptericin and Attacin mRNA is normalized with RP49. The data presented were mean±S.D. of 4 independent experiments.
peptides rapidly and massively. Two distinct pathways for the activation of the Drosophila immune response have been identified. Infection by Gram-negative bacterial pathogens leads to the production of antibacterial peptides, such as Attacin, Diptericin, and Cecropin, a function normally mediated by IKK-dependent activation of a NF-kappaB transcription factor, Relish [10]. TAK1 is essential for the PGN-induced expression of antimicrobial peptide genes by regulating NF-kappaB activation [14]. To examine whether dTAB2 is required for the expression of antimicrobial peptide genes, the S2* cells treated or not treated with dsRNA of dTAB2 were stimulated with commercial PGN-containing LPS for various times. The amount of mRNA for Diptericin and Attacin, two of antimicrobial peptides that are typical for IMD pathway, were measured by real-time RT-PCR. The copy number of Diptericin and Attacin mRNA was normalized with RP49. The results indicate that stimulation of PGN significantly induced the expression of Diptericin and Attacin in wild-type Drosophila cells, but not in dTAB2 “knocking-down” S2* cells (Fig. 6A and B), suggesting that dTAB2 is required for the NF-kappaB-mediated production of antimicrobial peptides in the innate immune response of Drosophila cells.

4. Discussion

The innate immune system of Drosophila responds to microbial infection by producing an array of antimicrobial peptides massively and rapidly [1-4]. Previous studies have demonstrated that Drosophila TAK1 is a key regulator of PGN-induced antimicrobial responses by activating IKK and JNK pathways [13-15]. In this report, we have identified a TAB1-binding protein in Drosophila, namely dTAB2, which can induce the activation of NF-kappaB signaling pathway. Furthermore, by using dsRNA interference, we have demonstrated that dTAB2 is required for PGN-induced activation of JNK MAP kinase, and the expression of antimicrobial peptide Diptericin and Attacin.

Studies in mammalian system have shown that activation of TAK1 is regulated by two TAB1 binding proteins, TAB1 and TAB2/3 [19-21]. So far, no TAB1-like proteins have been identified in Drosophila. However, in this report, we have identified a TAB2-like protein, namely dTAB2 in Drosophila. The dTAB2 appears to associate with dTAK1, thus dTAB2 also acts as a dTAK1-associating protein in Drosophila. In mammalian cells, TAB2 not only binds with TAK1, but also associates with an upstream regulator TRAF6 to form a TAK1–TAB2–TRAF6 complex [21-26]. TRAF6 contains an N-terminal RING domain, and functions as an ubiquitin ligase, which, in conjunction with a dimeric Ub-conjugating enzyme complex consisting of Ubc13 and Uev1A.Mms2, to catalyze the K63-linked ubiquitination of TRAF6. TAB2 binds to K63-linked polyubiquitinated TRAF6 though a highly conserved C-terminal zinc finger (ZnF) domain, thus facilitating the activation of TAK1. In Drosophila, TAB2 also contains a conserved ZnF domain at its C-terminal, and deletion of this region eliminated its ability to activate the NF-kappaB signaling pathway. In addition, two TRAF homologues, dTRAF1 and dTRAF2, have been identified in Drosophila. Only dTRAF2 contains a RING domain, and dTRAF2 mutant has a reduced level of Diptericin and Drosomycin induction after E. coli infection [34]. However, whether dTRAF2 can be polyubiquitinated and whether dTRAF2 binds with polyubiquitinated dTRAF2 to facilitate the activation of dTAK1 still requires further biochemical study.

In mammals, the physiological importance of TAB2 has been clarified by studies on TAB2-deficient mice and RNAi studies [25,27]. The phenotype generated from the TAB2 deficiency is very similar to that of NF-kappaB-, IKK-beta-, and NEMO/IKK-deficient mice. However, IL-1 or TNF-induced activation of NF-kappaB and JNK signaling pathways appears to be normal in TAB2-deficient embryonic fibroblasts. Recently, another mammalian TAB2 homologue, TAB3 has been identified, suppression of both TAB2 and TAB3 by RNAi inhibit the activation of IKK and JNK by IL-1 and TNF [25]. In Drosophila, the generation of dTAB2-deficient fly has not been reported so far. However, here we have shown that silencing of dTAB2 by using dsRNA inhibits PGN-induced activation of JNK and the expression of antibacterial peptide diptericin and attacin gene, two typical peptides produced when IMD signaling pathway are activated by Gram-negative bacterial pathogens (Fig. 7). Since the dTAB2 is associated with dTAK1 and TAB2 is believed to be a key regulator of TAK1, these results are consistent with previous studies that have shown that the Drosophila TAK1 is essential for the activation of both NF-kappaB and JNK MAP kinase signaling after PGN stimulation [13-15]. Considering the association of dTAB2 with dTAK1 and the functional role of its mammalian homologue TAB2 in the activation of TAK1, the physiological function of dTAB2 in the stimulation of PGN is most likely mediated through TAK1. On the other hand, sorbitol or NaCl-induced JNK activation was not impaired in the dTAB2-silenced Drosophila cells, which is also consistent with previous findings by Chen et al., which has shown that silencing of
TAK1 by dsRNAi does not inhibit the activation of JNK by sorbitol, and sorbitol-induced JNK activation is mediated through four MAP3Ks [13].

Compared to the studies of TAB2 and TAK1 in the activation of IKK and JNK, the functional role of TAB2 and TAK1 in the activation of p38 MAP kinase is less clear. TAK1 has been shown to directly phosphorylate and activate the MKK6, a MAP2K of p38 in vitro [19,21]. However, we did not observe any significant defect of PGN-induced p38 activation in TAB2-silenced Drosophila cells. In addition, our previous study has demonstrated that PGN-induced p38 activation is normal in TAK1-silenced cells [32]. These results suggest that TAK1 and its associating protein TAB2 may not be involved in p38 activation in PGN stimulation. The activation of p38 by TAK1 in previous reports may have been caused by TAB1 protein, another component of TAK1 activation complex [35,36].

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