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HTLV-1 Tax-mediated TAK1 activation involves TAB2 adapter protein

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Abstract

Human T cell leukemia virus type 1 (HTLV-1) Tax is an oncoprotein that plays a crucial role in the proliferation and transformation of HTLV-1-infected T lymphocytes. It has recently been reported that Tax activates a MAPKKK family, TAK1. However, the molecular mechanism of Tax-mediated TAK1 activation is not well understood. In this report, we investigated the role of TAK1-binding protein 2 (TAB2) in Tax-mediated TAK1 activation. We found that TAB2 physically interacts with Tax and augments Tax-induced NF-κB activity. Tax and TAB2 cooperatively activate TAK1 when they are coexpressed. Furthermore, TAK1 activation by Tax requires TAB2 binding as well as ubiquitination of Tax. We also found that the overexpression of TRAF2, 5, or 6 strongly induces Tax ubiquitination. These results suggest that TAB2 may be critically involved in Tax-mediated activation of TAK1 and that NF-κB-activating TRAF family proteins are potential cellular E3 ubiquitin ligases toward Tax.

Keywords: HTLV-1; Tax; NF-κB; TAK1; MAP3K; TRAF; Ubiquitination; Adapter protein

Human T cell leukemia virus type 1 (HTLV-1) is a retrovirus that causes adult T cell leukemia (ATL) [1]. In addition to the structural genes, HTLV-1 encodes a trans-acting regulatory protein, Tax, which is crucial for the transformation of infected cells [2]. The oncogenic action of Tax is associated with aberrant activation of cellular transcription factors, including the serum response factor (SRF), the cAMP-responsible element binding protein (CREB), and the nuclear factor-κB (NF-κB) [3]. Previous studies suggest that NF-κB activation induced by Tax is critical for the onset of ATL [4–7].

In unstimulated cells, NF-κB resides in the cytoplasm in an inactive form through its association with the IκB family of inhibitory proteins. Upon stimulation with cytokines, pathogens, or stress inducers, the IκB proteins are specifically phosphorylated followed by ubiquitination and degraded through a proteasome-dependent mechanism. The proteolysis of IκB releases NF-κB and allows it to translocate into the nucleus, where it activates the transcription of a large array of target genes [8]. The kinase responsible for the phosphorylation of IκB is known as the IκB kinase (IKK) complex, composed of two catalytic subunits, IKKα and IKKβ, and a regulatory subunit, NEMO (also known as IKKγ) [9].

Transforming growth factor-β-activated kinase 1 (TAK1) is a member of the MAPKKK family and is activated by various stimuli, including proinflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β), Toll-like receptor ligands, and T cell receptors [10]. Genetic and biochemical studies have demonstrated that TAK1 is a pivotal kinase that activates the IKK complex as well as the p38 and c-Jun N-terminal kinase (JNK) MAP kinase pathway in response to these stimuli [11–15]. Cytokine-induced TAK1 activation requires its binding protein TAB2 and its homologue TAB3, which link TAK1 to the upstream signaling molecule TRAF family proteins [16–18]. TAB2 and TAB3 facil-

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itate the formation of a complex of TAK1 with TRAFs and thereby activate TAK1.

It has recently been reported that Tax activates TAK1 either by its physical interaction [19] or by inducing the overexpression of TAB2 [20]. However, the precise molecular mechanism of Tax-mediated TAK1 activation is not well understood. In this study, we examine the role of TAB2 in the Tax-mediated activation of TAK1. We demonstrate that Tax activates TAK1 in a manner dependent on its physical association with TAB2 as well as Tax ubiquitination. Moreover, we found that TRAF2, 5, or 6 may function as cellular E3 ubiquitin ligases toward Tax.

Materials and methods

Reagents, expression vectors, and cell culture. Anti-FLAG monoclonal antibody M2 (Sigma), anti-T7 monoclonal antibody (Novagen), anti-HA monoclonal antibody HA.11 (Covance), anti-6xHis monoclonal antibody AD1.1.10 (R&D Systems), and anti-TAK1 polyclonal antibody M-579 (Santa Cruz) were used. Expression vectors for FLAG-TAB2 (FL, 1–693 a.a.; N, 1–400 a.a.; C, 401–693 a.a.; and ΔCUE, 54–693 a.a.) and FLAG-TAK1 (full-length, 1–597 a.a.; ΔC, 1–500 a.a.) were constructed in this study. Cloning details are available upon request. Wild-type (WT) and lysine-less (KR) mutant Tax cDNA (kindly provided by Dr. Claudine Pique, Cochin Institute) [21] were subcloned into a pCMVT7 vector to obtain expression of the C-terminal T7 epitope-tagged Tax proteins. The expression vectors for Tax-His and FLAG-TRAF proteins were described previously [21,22]. 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), L-glutamine (200 μM), penicillin G (50 U/ml), and streptomycin (50 μg/ml). For the transfection studies, cells (1×10^6) were plated in 6-cm dishes and transfected using linear 25-kDa polyethyleneimine (PEI, Polysciences, Inc.) by combining 5 μg of plasmid DNA with 15 μl of PEI (1 mg/ml) in 0.5 ml of serum free DMEM for 15 min prior to addition to cells [23].

Immunoprecipitation and Western-blotting. Cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed in 0.3 ml of 0.5% Triton X-100 lysis buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, 1.5 mM $MgCl_2$, 12.5 mM β-glycerophosphate, 10 mM NaF, and 1 mM sodium orthovanadate supplemented with protease inhibitor cocktail (Nacalai Tesque). Cellular debris was removed by centrifugation at 15,000*g* for 5 min. Proteins from cell lysates were immunoprecipitated with 1 μg of an antibody and 20 μl of Protein G-Sepharose (GE Healthcare) for 2 h at 4 °C. The immune complex was washed three times with washing buffer containing 20 mM Hepes (pH 7.4), 500 mM NaCl, and 10 mM $MgCl_2$ and was suspended in 40 μl of rinse buffer containing 20 mM Hepes (pH 7.4), 150 mM NaCl, and 10 mM $MgCl_2$. For Western-blotting, the immunoprecipitates or whole cell lysates were resolved on SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to Immobilon-P membranes (Millipore). The membranes were Western-blotted with various antibodies, and the bound antibodies were visualized with horseradish peroxidase-conjugated antibodies against rabbit or mouse IgG using Chemi-Lumi One L Western-blotting detection reagents (Nacalai Tesque).

In vitro phosphorylation assay. Anti-FLAG immunoprecipitates were incubated with 1 μg of bacterially expressed MKK6 in 10 μl of kinase buffer containing 10 mM Hepes (pH 7.4), 1 mM DTT, 5 mM $MgCl_2$, and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) at 25 °C for 5 min. Samples were separated by 10% SDS–PAGE and visualized by autoradiography.

Ni–NTA-agarose pull-down. Nickel–nitrilotriacetic acid (Ni–NTA)-agarose affinity pull-down experiments were performed according to the manufacturer's instructions (Qiagen). Briefly, at 24 h post-transfection, 293T cells were lysed under reducing and highly denaturing conditions and incubated with Ni–NTA-agarose beads for 2 h at 4 °C. The beads were washed extensively, and the bound proteins were eluted.

Reporter gene assay. For the reporter gene assays, 293T cells (2×10^5 cells/well) were seeded into 6-well (35 mm) plates. At 24 h after seeding, cells were transfected with a reporter plasmid and expression plasmids as indicated. An Ig-κ-luciferase reporter was used to measure NF-κB-dependent gene activation. A plasmid containing the β-galactosidase gene under the control of the β-actin promoter (pAct-β-Gal) was used to normalize for transfection efficiency.

Results and discussion

TAB2 is a Tax-binding protein

It has recently been shown that HTLV-1 Tax oncoprotein activates TAK1 [19,20]. However, the molecular mechanism for Tax-mediated TAK1 activation is not well understood. Since TAB2 acts as an adapter to activate TAK1 in the cytokine signaling pathway, we hypothesized that TAB2 may also be involved in TAK1 activation by Tax. First, to examine whether TAB2 physically interacts with Tax, we performed co-immunoprecipitation experiments in 293T cells transiently transfected with T7-tagged Tax and FLAG-tagged TAB2 (Fig. 1). Cell extracts were immunoprecipitated with anti-FLAG antibody, and coprecipitated Tax-T7 was detected by Western-blotting with anti-T7 antibody. TAB2 was found to associate with Tax. To determine the Tax-binding region in TAB2, its truncated mutants were tested for their interaction with Tax. As shown previously [18], TAK1 interacted with the C-terminus (amino acids 401 to the end) of TAB2 (lane 4). In contrast, Tax was found to associate with the N-terminus

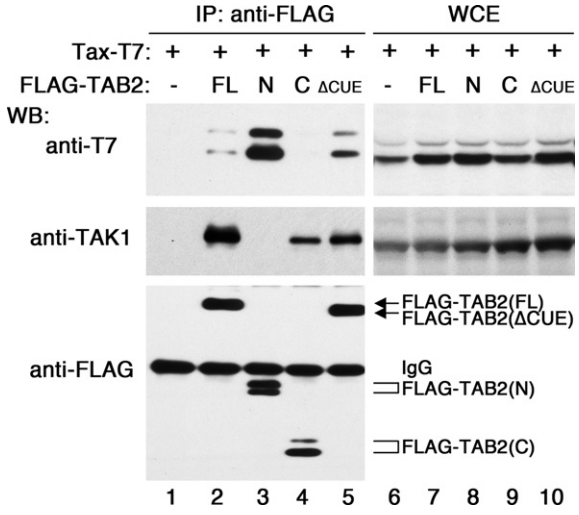

Fig. 1. TAB2 interacts with Tax. 293T cells were transiently transfected with an expression vector for Tax-T7 in combination with an empty vector (−) or with expression vectors for FLAG-TAB2 full-length (FL), FLAG-TAB2N (N), FLAG-TAB2C (C), or FLAG-TAB2ΔCUE (ΔCUE), as indicated. Cell extracts were immunoprecipitated with anti-FLAG. Coprecipitated Tax-T7 and endogenous TAK1 were detected by Western-blotting with anti-T7 (left, top panel) and anti-TAK1 (left, middle panel), respectively. The amounts of immunoprecipitated FLAG-TAB2 proteins were determined with anti-FLAG (left, bottom panel). Whole cell extracts were Western-blotted to determine the total amounts of Tax-T7 and TAK1 (right panels).

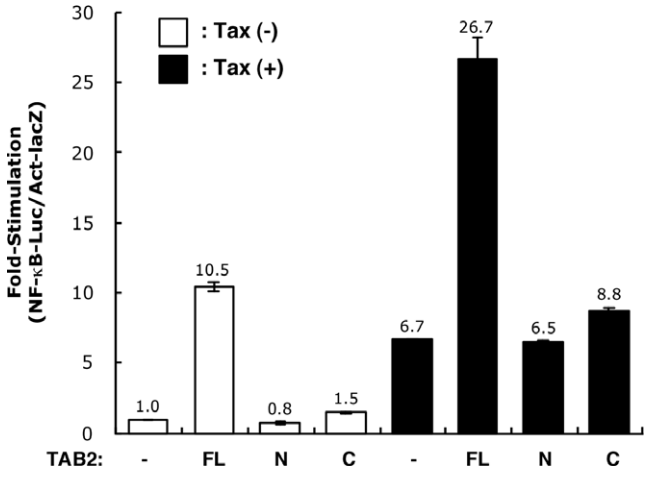

Fig. 2. TAB2 and Tax cooperatively activate NF-κB. 293T cells were transiently transfected with the reporter vector Ig-κ-Luciferase (100 ng) and pAct-β-Gal (50 ng) along with an empty vector (−) or 150 ng of expression vectors for TAB2 full-length (FL), TAB2N (N), or TAB2C (C) in the absence (open bars) or the presence (solid bars) of 500 ng of Tax expression plasmid. The luciferase activities were determined and normalized to the levels of β-galactosidase activity. The results are expressed as the fold-increase in luciferase activity relative to cells transfected with an empty vector.

(amino acids 1–400) of TAB2 (lane 3). The N-terminus of TAB2 contains a ubiquitin-binding motif called the CUE domain [24]. We tried to determine whether this motif is required for the interaction with Tax and found that the CUE domain is dispensable for interaction with Tax (lane 5). These results suggest that TAB2 can form a complex with Tax via a different region from that required for TAK1 binding.

Tax and TAB2 cooperatively activate NF-κB

One of the cellular functions of Tax is the activation of the NF-κB pathway. To investigate the functional relationship between TAB2 and Tax, we examined the effect of TAB2 overexpression on Tax-induced NF-κB activity using an NF-κB-dependent luciferase reporter (Fig. 2). As previously demonstrated [25], the expression of Tax leads to the activation of NF-κB in 293T cells. The overexpression of full-length TAB2, which also activates NF-κB by itself, enhanced Tax-induced NF-κB activity, while neither the N-terminal nor the C-terminal half of TAB2 had such an effect. Therefore, TAB2 can function as an augmenting factor for Tax, presumably by forming a ternary complex with TAK1 and Tax.

Tax activates TAK1 in concert with TAB2

The results above indicate that Tax and TAB2 may coordinate in activating TAK1. To test this possibility, we examined the activity of TAK1 coexpressed with Tax and/or TAB2 by an *in vitro* kinase assay using bacterially expressed His-MKK6 protein as a substrate (Fig. 3). Although TAK1 kinase activity was only slightly increased by coexpression with Tax (lane 3), TAK1 was strongly acti-

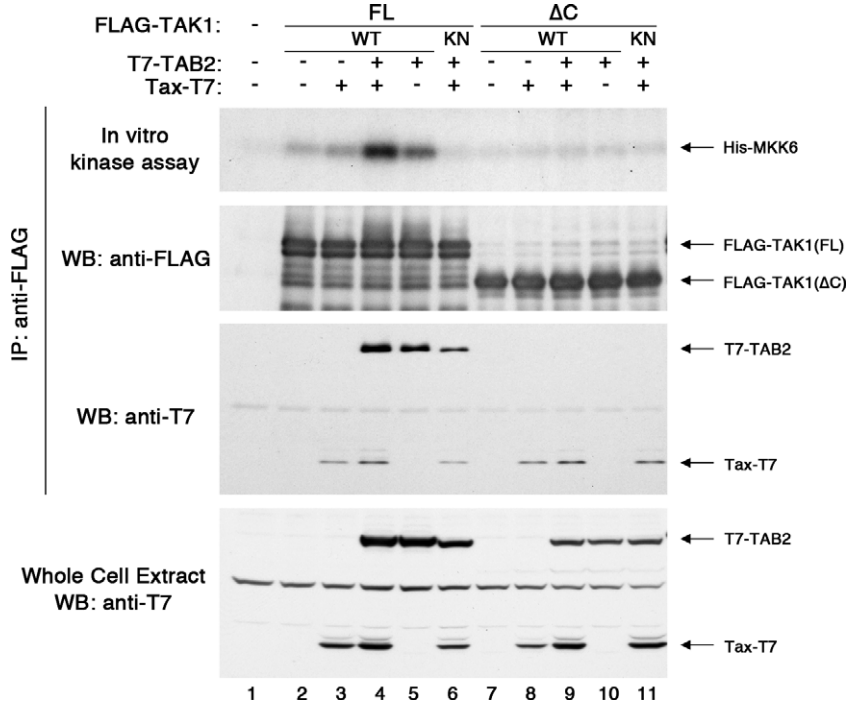

Fig. 3. Tax activates TAK1 in concert with TAB2. 293T cells were transiently transfected with expression vectors for FLAG-TAK1 full-length (FL) or FLAG-TAK1ΔC (ΔC) (wild-type or kinase-negative mutant for each construct indicated as WT or KN, respectively), T7-TAB2 and Tax-T7, as indicated. Immunoprecipitated complexes with anti-FLAG were subjected to an *in vitro* kinase assay with bacterially expressed His-MKK6 as a substrate (top panel) or Western-blotted with anti-FLAG (second panel). The amounts of T7-TAB2 and Tax-T7 in immunoprecipitates and whole cell extracts were also determined by Western-blotting with anti-T7 (third and bottom panels, respectively).

vated by the simultaneous expression of Tax and TAB2 (lane 4) even at a higher level than by TAB2 alone (lane 5). Therefore, it is likely that Tax utilizes TAB2 to achieve the efficient activation of TAK1.

We then addressed the question of whether Tax-mediated TAK1 activation requires interaction with TAB2. We used a TAK1 mutant lacking its C-terminal 79 amino acids necessary for TAB2 binding. The mutant TAK1,

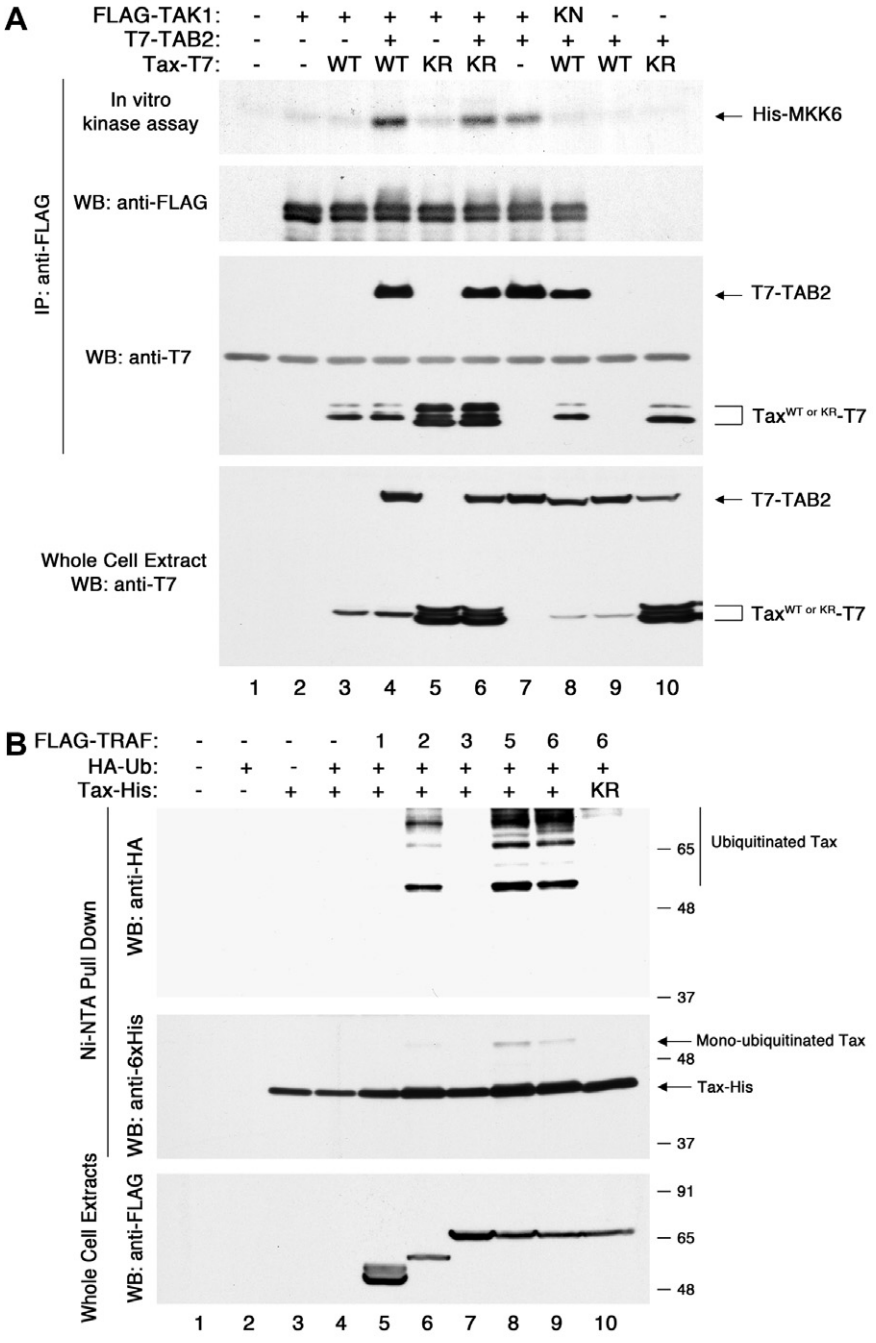

Fig. 4. TRAF family protein can induce the ubiquitination of Tax. (A) 293T cells were transiently transfected with expression vectors for FLAG-TAK1 [wild-type (+) or kinase-negative (KN)], T7-TAB2 and Tax-T7 [wild-type (WT) or lysine-less mutant (KR)], as indicated. *In vitro* kinase assay and Western-blotting were performed as described in Fig. 3. (B) 293T cells were transiently transfected with expression vectors for Tax-His [wild-type (WT) or lysine-less mutant (KR)], HA-Ubiquitin (Ub), and FLAG-TRAF proteins (1, 2, 3, 5, or 6), as indicated. The cells were harvested 24 h post-transfection and divided into two sets; one set was used for Ni–NTA pull-down assay, while the other set was used for preparation of whole cell extracts. Cell extracts prepared under reducing and highly denaturing conditions were subjected to Ni–NTA-agarose affinity pull-down. The eluates from Ni–NTA bead pull-down experiments were separated in SDS–PAGE and Western-blotted with anti-HA (top panel) or anti-6xHis (middle panel). Whole cell extracts prepared in non-denaturing lysis buffer were Western-blotted to determine the amounts of FLAG-TRAF proteins (bottom panel). The positions of the protein standards are shown on the right of each panel.

TAK1ΔC, is unable to interact with TAB2 (Fig. 3, lanes 9–11) but retains an intact kinase domain that can be activated by the overexpression of TAB1 ([26], and data not shown). Unlike full-length TAK1, TAK1ΔC was not activated by the coexpression of Tax and TAB2, although it was capable of binding with Tax (lane 9), indicating that Tax-mediated activation of TAK1 requires the interaction with TAB2.

TRAF family proteins are potential cellular E3 ubiquitin ligases toward Tax

Previous studies demonstrated that Tax is ubiquitinated in mammalian cells and this ubiquitination is essential for its function, such as the activation of NF-κB [21,27–29]. We, therefore, sought to determine whether the ubiquitination of Tax is necessary for its ability to activate TAK1. As shown in Fig. 4A, wild-type Tax activated TAK1 in concert with TAB2 (compare lanes 4 and 7). In contrast, a lysine-less mutant (Tax^{KR}), in which all the lysine residues are substituted with arginines, resulting in the loss of its ability to be ubiquitinated, failed to do so (compare lanes 6 and 7). Therefore, it is likely that the ubiquitination of Tax is required for the activation of TAK1.

To date, the cellular E3 ubiquitin ligase(s) responsible for the ubiquitination of Tax have not yet been identified. Since TRAF family proteins possess E3 ubiquitin ligase activity and function in proximity to TAK1, we hypothesized that some of them could be such candidates. The Tax protein fused to a C-terminal six-histidine tag (Tax-His) was expressed in 293T cells along with HA-tagged ubiquitin and FLAG-tagged TRAF1, 2, 3, 5, or 6. Cells were lysed under reducing and highly denaturing conditions, and a Ni–NTA pull-down assay was performed. Ubiquitinated Tax was marginally detected when Tax-His and HA-Ub were coexpressed (lane 4). On the other hand, cotransfection of TRAF2, 5, or 6, but not 1 or 3, strongly stimulated the ubiquitination of Tax (lanes 5–9), whereas the Tax^{KR} mutant was not ubiquitinated even in the presence of TRAF6 (lane 10). These results indicate that NF-κB-activating TRAF family proteins are capable of inducing the ubiquitination of Tax.

In summary, we have shown here that Tax physically interacts with TAB2 and accomplishes the efficient activation of TAK1. We also demonstrated that the ubiquitination of Tax is required for the Tax-mediated activation of TAK1 and that NF-κB-activating TRAF family proteins, such as TRAF2, 5, or 6, could be potential cellular E3 ubiquitin ligases toward Tax. However, an ubiquitination-defective Tax mutant was still capable of binding to TAB2 as well as TAK1, and the CUE domain was dispensable for TAB2 to interact with Tax. Therefore, although the ubiquitination of Tax seems to be necessary for TAK1 activation, the precise mechanism of how Tax ubiquitination increases TAK1 activity currently remains unclear. Further analysis is needed to elucidate the exact role of ubiquitination in the Tax-mediated activation of TAK1.

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