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TGFβ-activated kinase 1 (TAK1)-binding proteins (TAB) 2 and 3 negatively regulate autophagy

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Transforming growth factor β-activated protein kinase 1 (TAK1)-binding protein 2 (TAB2) and its close homolog TAB3 are initially characterized as adapter proteins essential for TAK1 activation in response to interleukin-1β and tumour necrosis factor-α. However, the physiological roles of TAB2 and TAB3 are still not fully understood. Here we report that TAB2 and TAB3 bind to Beclin1 and colocalize in the cytoplasm. TAB2 also interacts with ATG13 and is phosphorylated by ULK1. Overexpression of TAB2 or TAB3 induces punctate localization of ATG5 under the normal culture condition. Knockdown of TAB2 and TAB3 results in the decrease in endogenous protein level of p62/SQSTM1 under the normal culture condition, while overexpression of TAB2 results in the accumulation of p62/SQSTM1 independently of TAK1. The decrease of p62/SQSTM1 induced by the knockdown of TAB2 and TAB3 is largely dependent on ATG5. These results suggest that TAB2 and TAB3 negatively regulate autophagy independently of TAK1 activity.

Keywords: autophagy/Beclin1/TAK1-binding proteins.

Abbreviations: ATG, autophagy-related genes; Bcl-2, B-cell lymphoma/leukaemia-2; IL-1β, interleukin-1β; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase; MEFs, mouse embryonic fibroblasts; PBS, phosphate-buffered saline; PE, phosphatidylethanolamine; PEI, polyethyleneimine; PI3K, phosphatidylinositol 3-kinase; TAK1, transforming growth factor β-activated kinase 1; TAB2 and TAB3, TAK1-binding proteins 2 and 3; TNF-α, tumour necrosis factor-α; TRAIL, TNF-related apoptosis-inducing ligand; TRAF, TNF receptor-associated factor; ULK, unc51-like kinase.

Macroautophagy (hereafter referred to as autophagy) is an evolutionarily conserved intracellular bulk degradation system. During autophagy, a portion of the cytoplasm is engulfed by a double-membraned vesicle called autophagosome and delivered to the lysosome to be degraded. In mammals, there are roughly two types of autophagy: 'induced autophagy' and 'basal (or constitutive) autophagy'. The former is strongly induced upon metabolic or genotoxic stresses primarily as a cell survival response, while the latter occurs constitutively at low level for turnover of cytosolic components. Recent studies have revealed the critical roles of autophagy under physiological and pathological conditions including neurodegeneration, liver degeneration and immune response (*1–3*). Although autophagy has generally been considered as a non-selective degradation system, accumulating evidence suggests that ubiquitin-positive substrates, such as protein aggregates, mitochondria and intracellular bacteria, are selectively targeted for autophagic degradation via adapter proteins that interact with both ubiquitinated targets and the autophagic machinery (*4*).

Approximately 30 autophagy-related (ATG) genes have been identified in yeast, and 18 of which compose the core machinery of autophagosome formation (*2*). These Atg proteins can be classified into five subgroups: Atg1 [unc51-like kinase (ULK)] complex, Vps34 class III phosphatidylinositol 3-kinase (PI3K) complex, the Atg9 [mAtg9] cycling complex and two ubiquitin-like protein (Atg12 and Atg8 [LC3]) conjugation systems (*5*). Mammalian ULKs (ULK1 and 2) form a complex with ATG13, FIP200 and ATG101, and the kinase activity of ULK1 is important for autophagy (*6–8*). Although ULK1 can phosphorylate ATG13 and FIP200 (*9*, *10*), its physiological substrates are currently unknown. Vps34 class III PI3K associates with Atg6 [Beclin1] and is found in at least three different complexes in mammals, whereby regulates autophagy at multiple levels (*11*, *12*). Atg8 [LC3] exists in two forms: the cytosolic form (LC3-I) and the lipidated form (LC3-II) which is conjugated to phosphatidylethanolamine (PE) and specifically localizes on the elongating and mature autophagosomes (*13*). Atg12 forms a stable conjugate with Atg5 and interacts non-covalently with Atg16L, which acts as an E3-like enzyme essential for Atg8 [LC3]-PE conjugation reaction (*14*). Besides the well-conserved core machinery, a number of genes are found to regulate autophagy directly or indirectly in mammals, such as signal mediators for growth factors, ER stress, hypoxia, oxidative stress and so on (*3*). For example, the function of Beclin1 is negatively regulated by B-cell lymphoma/leukemia-2 (Bcl-2) through direct binding and sequestering Beclin1 under nutrient-rich conditions (*15*). However, the molecular mechanisms for autophagy regulation in such diverse biological processes in mammalian cells are not fully understood.

Transforming growth factor β-activated kinase 1 (TAK1)-binding proteins 2 and 3 (TAB2 and TAB3) are ubiquitously expressed and redundantly play essential roles in activation of TAK1 in response to inflammatory cytokines such as interleukin-1β (IL-1β) and tumour necrosis factor-α (TNF-α) (*16–18*). Upon stimulation, TAB2 and TAB3 link TAK1 to the upstream signalling molecules TNF receptor-associated factor (TRAF) proteins, thereby mediate the activation of TAK1. Activated TAK1 in turn, activates p38 and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinase (MAPK) cascade as well as nuclear factor-κB (NF-κB) signalling pathway (*19*). To date, the *in vivo* roles of TAK1 and TAB2 have been examined using gene knockout mice. TAK1 knockout mice die around E9.5 during development with defects in neural tube formation and vascular development (*20–22*), while TAB2 knockout mice die around E12.5 with massive liver apoptosis, suggesting that TAK1 and TAB2 are essential for embryogenesis (*23*). In addition, the mice with hepatocyte-specific gene deletion of TAK1 are viable at birth, but show spontaneous apoptosis and subsequent compensatory liver cell proliferation, which eventually results in carcinogenesis in the liver (*24*). These results indicate that TAB2 may have TAK1-independent functions in liver cell survival during embryogenesis.

To identify previously unknown function(s) of TAB2 and TAB3, we conducted a yeast two-hybrid screening and isolated Beclin1 as a new TAB2- and TAB3-binding protein. We found that TAB2 and TAB3 negatively regulate basal level autophagy independently of TAK1 activity.

Experimental procedures

Yeast two-hybrid screening

A cDNA encoding full-length human TAB3 was fused to the Gal4 DNA-binding domain in the pGBD-C1 vector (pGBD-TAB3). The yeast strain PJ69-4A was sequentially transformed with pGBD-TAB3 and a library containing mouse embryo cDNAs fused to the Gal4 activation domain by standard lithium acetate method. Approximately 2.2×10^5 transformants were obtained and screened as described previously (*25*). Two independent clones encoding amino acids 48–448 and 69–448 of Beclin1 were isolated.

Cell culture and reagents

293T, MCF-7 cells and mouse embryonic fibroblasts (MEFs) including TAK1-WT (wild-type), TAK1-Δ (deficient) and m5-7 were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, GlutaMAX I (GIBCO) and non-essential amino acids (GIBCO) in a 5% CO_2 incubator at 37°C. MCF-7 cells were obtained from RIKEN Cell Bank (Cell No. RCB1904). 293T cells were described previously (*26*). TAK1-WT and TAK1-Δ MEFs (*20*) and m5-7 cells (*27*) were kind gifts from Drs Shizuo Akira and Noboru Mizushima, respectively.

Plasmids, siRNAs and transfection

To obtain the full-length mouse Beclin1 cDNA, the N-terminal portion encoding amino acids 1–182 was cloned by RT-PCR using total cDNA from wild-type MEF as a template. The following primers were used; 5′-gaattcatggaggggtctaaggcgtccagc-3′ and 5′-cagctccct ctgcagctgctcact-3′. The full-length mouse Beclin1 cDNA was generated by ligation of an EcoRI-PstI fragment of the Beclin1 N-terminal cDNA and a PstI-SalI fragment of the Beclin1 cDNA from the library clone isolated in the screening. This full-length mouse Beclin1 cDNA was subcloned into mammalian expression vectors, pCMV-FLAG and pCMV-EGFP. The full-length human TAB2 and TAB3 cDNA (*17*, *18*) were subcloned into pCMV-DsRedMonomer-T7 vector to generate pCMV-DsRedMT7-TAB2 and pCMV-DsRedMT7-TAB3, respectively. The expression vector for EGFP-ATG5, FLAG-ULK1(WT and KN), FIP200 and ATG13 were kindly provided by Drs Tamotsu Yoshimori and Noboru Mizushima (*7*, *9*, *28*). 293T cells were transfected using polyethyleneimine (PEI, Polysciences) transfection method (*29*). For transfection into MCF-7 or MEFs, FuGENE HD transfection reagent (Roche) was used according to the manufacturer's instruction. For knockdown experiments, following siRNAs were used: siTAB2-#1, 5′-GCAUGGGUCCUGCCUUUAU-3′, 5′-CGUACC CAGGACGGAAAUA-3′; siTAB2-#2, 5′- GCACAUGUGGAUA GAAUAA-3′, 5′-CGUGUACACCUAUCUUAUU-3′; siTAB3-#1, 5′-GCUCAAGUGAUGGACAUAU-3′, 5′-CGAGUUCACUACC UGUAUA-3′; siTAB3-#2, 5′- GCGUCCUUUACCUGUUUA U-3′, 5′-CGCAGGAAAUGGACAAAUA-3′; siTAB3-#3, 5′-GCU GCAGAUGAACAUUUAA-3′, 5′-CGACGUCUACUUGUAAA UU-3′; siCTRL, 5′- UUCUCCGAACGUGUCACGU-3′, 5′-AAG AGGCUUGCACAGUGCA-3′. MEF or MCF-7 cells were reverse transfected with siRNAs at the final concentration of 50 pmol/ml (25 pmol/ml each for the double knockdown) using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

Immunoprecipitation and western blotting

Cells were washed once with ice-cold phosphate-buffered saline (PBS) and lysed in lysis buffer containing 20 mM HEPES pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EGTA, 0.5 mM dithiothreitol (DTT) and 2% Triton X-100 supplemented with protease inhibitor cocktail and phosphatase inhibitor cocktail (Nacalai Tesque). Proteins from cell extracts were immunoprecipitated with various antibodies and protein G Sepharose (GE Healthcare) for 2 h at 4°C. For western blotting, immunoprecipitates or whole-cell extracts were resolved by SDS–PAGE and transferred to Immobilon-P Polyvinylidene fluoride (PVDF) membranes (Amersham). The membranes were incubated with various antibodies, and the bound antibodies were visualized with horseradish-peroxidase (HRP)-conjugated antibodies against rabbit or mouse IgG using the Chemi-Lumi One L Chemiluminescence western blotting reagent (Nacalai Tesque). The following antibodies were used: monoclonal antibodies against T7-tag (#69522, Novagen), FLAG-tag (M2, SIGMA), ATG5 (M153-3, MBL) and α-tubulin (DM1A, SIGMA); polyclonal antibodies against TAB2 (*18*), TAK1 (*19*), p62/SQSTM1 (PM045, MBL), LC3 (PM036, MBL), cleaved caspase-3 (#9661, Cell Signaling) and β-actin (A2066, SIGMA).

Quantitative RT-PCR

Cells were washed once with ice-cold PBS and pelleted. Total RNA was isolated using the TRIzol reagent (Life Technologies) and reverse transcribed into cDNA by MultiScribe reverse transcriptase using random hexamer (Applied Biosystems). Quantitative real-time PCR was performed on ABI PRISM 7000 using the SYBR Green PCR Master Mix (Applied Biosystems). The following primers were used: mTAB2-forward, 5′-GGATAGAATAAGCGAAGCCC GGAA-3′; mTAB2-reverse, 5′-CTCTTTGAAGCCGTTCCATCCT-3′; mTAB3-forward, 5′-CAGAGACGGCTTAGAAGAGT-3′; mTAB3-reverse, 5′-GGAGGTCAACTTCTTTCAGTGT-3′; mβActin-forward, 5′-CCCAGAGCAAGAGAGGTATC-3′ and mβActin-reverse, 5′-AG AGCATAGCCCTCGTAGAT-3′.

Phos-tag

Phos-tag technique (*30*) was used to detect phosphorylated protein according to the manufacturer's instruction. In brief, T7-tagged TAB2, TAB3 or ATG13 were immunoprecipitated from whole-cell extract of the transfected cells, separated by SDS–PAGE and transferred onto PVDF membrane. The membrane was then incubated with the mixture of biotin-conjugated Phos-tag (Phos-tg BTL, NARD) and HRP-conjugated streptavidin (eBioscience) for 30 min at room temperature. After washing, the bound Phos-tag BTL was visualized using the Chemi-Lumi One Super western blotting reagent (Nacalai Tesque).

Laser scanning confocal microscopy

MCF-7 cells grown on coverslips were transiently transfected with expression plasmids for EGFP-Beclin1 together with DsRed-Monomer ($DsRed^M$)-TAB2, TAB3 or empty vector.

Twenty four hours after transfection, cells were washed once with PBS and fixed with 4% paraformaldehyde/PBS for 15 min. Then, the coverslips were washed three times with PBS and mounted in Permafluor fluorescent mounting medium (Beckman Coulter). A Zeiss LSM510 META laser scanning confocal microscope (Carl Zeiss) was used to image cells (Nikon Plan Apo, 60x NA1.4 oil immersion lens). The pinholes were set to capture an optical slice of ~1.0 μm.

Results

TAB2 and TAB3 interact with Beclin1

To uncover previously unknown functions of TAB2 and TAB3, we sought to identify proteins that interact with the full length of TAB3 by yeast two-hybrid screening. Two independent clones that encode amino acids 48–448 and 69–448 of Beclin1 were isolated from mouse embryonic library. The interaction of TAB3 with Beclin1 in mammalian cells was confirmed by coimmunoprecipitation experiment in 293T cells, and the carboxyl terminal region of TAB3 containing a coiled-coil motif was found to be necessary and sufficient for Beclin1 binding (Fig. 1A and B). Since TAB2 and TAB3 are known to function redundantly in the IL-1β and TNF-α signalling pathway, we also tested the interaction of Beclin1 with TAB2 and found that TAB2 also interacted with Beclin1 in a similar manner as TAB3 did (Fig. 1C).

TAB2 and TAB3 colocalize with Beclin1

We next analysed the subcellular distribution of Beclin1, TAB2 and TAB3. Consistent with the previous reports, EGFP-Beclin1 evenly distributed throughout the cytoplasm (Fig. 2, top panel, EGFP) (*31*), and overexpressed TAB2 or TAB3 showed punctate localization pattern in the cytoplasm (Fig. 2, middle and bottom panels, $DsRed^{M}$) (*32*). We found that a portion of EGFP-Beclin1 colocalized with either $DsRed^{M}$-TAB2 or $DsRed^{M}$-TAB3 when they are expressed together (Fig. 2, middle and bottom panels, merged and magnified), further confirming the interaction of Beclin1 with TAB2 and TAB3 in mammalian cells. The ability of TAB2 and TAB3 to alter the subcellular localization of Beclin1 indicates that they may be involved in the biological processes regulated by Beclin1, such as autophagy.

Overexpression of TAB2 or TAB3 induces punctate localization of EGFP-ATG5

The above results prompted us to investigate the potential roles of TAB2 and TAB3 in autophagy regulation. We first tested whether overexpression of TAB2 or TAB3 could affect the subcellular localization of EGFP-tagged ATG5 protein, which is known to localize on the immature autophagosome called isolation membrane (also referred to as phagophore) during autophagy (*33*). As shown in Fig. 3, EGFP-ATG5 evenly distributed throughout the cytoplasm in MCF7 cells under a normal culture condition (Fig. 3, top panel). We observed a punctate localization of EGFP-ATG5 when it was coexpressed with $DsRed^{M}$-TAB2. In addition, overexpressed TAB2 protein was found to colocalize with ATG5 (Fig. 3, second panels). Similar result was observed by coexpression of TAB3

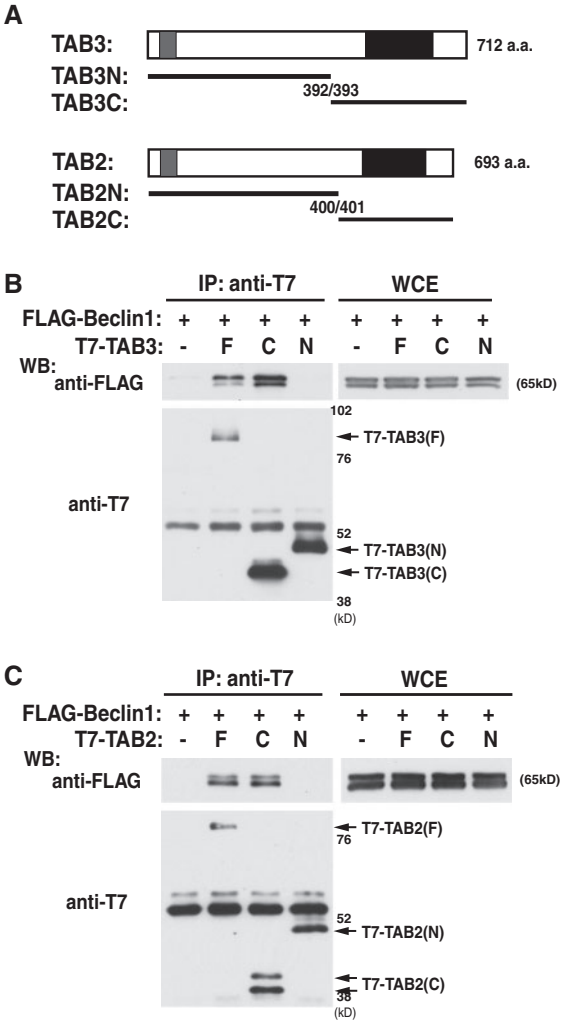

Fig. 1 TAB2 and TAB3 interact with Beclin1 in mammalian cells. (A) Schematic representation of TAB2 and TAB3 proteins, and their deletion constructs. The monoubiquitin-binding CUE domains and the coiled-coil motifs are shown as grey and black boxes, respectively. The regions encoded by their deletion constructs are shown as lines with the site of truncation indicated by the position of amino acids. (B) 293T cells were transiently transfected with expression plasmids for FLAG-Beclin1 along with T7-TAB3 (F, 1–712 aa; N, 1–392 aa; or C, 393–712 aa) or empty vector (−) as indicated. Twenty four hours after transfection, cell extracts were prepared and immunoprecipitated with anti-T7 antibody (IP), and analysed by western blotting with indicated antibodies (WB). Whole-cell extracts (WCE) were also western blotted to monitor the amount of protein in the extracts. (C) Coimmunoprecipitation assay was performed in a similar procedure except using T7-TAB2 (F, 1–693 aa; C, 401–693 aa; or N, 1–400 aa) instead of TAB3.

and ATG5 (data not shown), suggesting that TAB2 and TAB3 are likely to regulate autophagy in the vicinity of isolation membrane. We then asked whether the alteration of ATG5 localization by TAB2 requires its binding to Beclin1. We found that TAB2N itself showed a diffuse cytoplasmic localization and it had little effect on the localization of ATG5 (Fig. 3, third panels). TAB2C was expressed in perinuclear regions with a dot-like pattern, but the punctate localization of ATG5 was not observed (Fig. 3, bottom panels). Therefore, the binding to Beclin1 is necessary but not

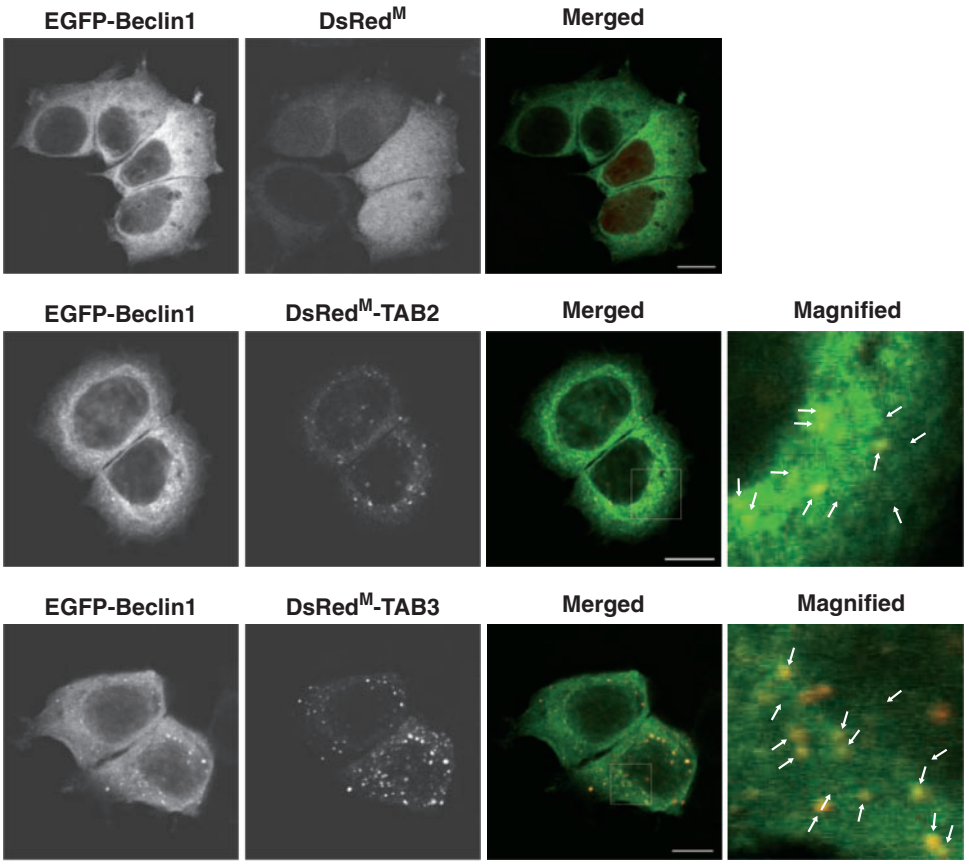

Fig. 2 TAB2 and TAB3 colocalize with Beclin1. MCF-7 cells were transiently transfected with EGFP-Beclin1 together with DsRed-Monomer (DsRed^{M}) or DsRed^{M}-fused TAB2 or TAB3 as indicated. After transfection, cells were cultured in DMEM for 24 h and fixed using 4% paraformaldehyde. Fluorescent images were obtained with Carl Zeiss LSM510 confocal microscope. Squares in the pictures indicate the area shown in the magnified view. Arrows indicate colocalization of Beclin1 and either TAB2 or TAB3. The pinholes were set to capture an optical slice of ~1.0 μm. Scale bar = 10 μm.

sufficient for the autophagy regulation by TAB2 and TAB3.

TAB2 and TAB3 inhibit basal autophagy

Because ATG5 protein is known to localize on the isolation membrane throughout its elongation process but dissociate from it once autophagosome is formed (*33*), there are two possibilities for the role of TAB2 and TAB3 in autophagy regulation: inducing the formation of isolation membrane (*i.e.* stimulating autophagy activity) or inhibiting the maturation of autophagosome (*i.e.* suppressing autophagy activity). To investigate these possibilities, we examined the effect of knockdown of TAB2 and/or TAB3 on autophagy activity assessed by the protein level of endogenous p62/SQSTM1, a known autophagy substrate (*34*). Because antibodies for the detection of endogenous TAB3 were not available to us, knockdown efficiency of siRNAs used in this study was evaluated by quantitative RT-PCR (Fig. 4A). Although single knockdown of either TAB2 or TAB3 in MEF had little effect on the level of p62/SQSTM1, simultaneous knockdown of both TAB2 and TAB3 markedly reduced the amount of p62/SQSTM1 under the normal culture condition (Fig. 4B). Similar results were obtained when human breast cancer cell line MCF-7 was transfected with the above siRNAs that are compatible with human TAB2 or TAB3 (Fig. 4C).

To eliminate a possibility that the loss of TAB2 and TAB3 may result in destabilization of p62/SQSTM1 independently of autophagy activity, we utilized ATG5 knockout MEFs reconstituted with the ATG5 gene under the control of the Tet-off expression system (m5-7 cells, (*27*)). As reported previously (*27*), more than 4 days of Doxycycline (Dox) treatment of these cells shut off ATG5 expression and completely blocked the autophagy activity as confirmed by the absence of LC3-II, another well-established autophagy indicator. Under this condition, knockdown of TAB2 and TAB3 only slightly reduced the amount of p62/SQSTM1 as compared to the cells cultured in the absence of Dox throughout the experiment (Fig. 4D), indicating that the decrease in p62/SQSTM1 protein level by knockdown of TAB2 and TAB3 is largely dependent on autophagy activity. Therefore, it is likely that both TAB2 and TAB3 negatively regulate basal autophagy.

TAB2 and TAB3 inhibit basal autophagy independently of TAK1 activity

We then examined the effect of overexpression of TAB2 on autophagy activity. In contrast to the knockdown experiments, overexpression of the full-length

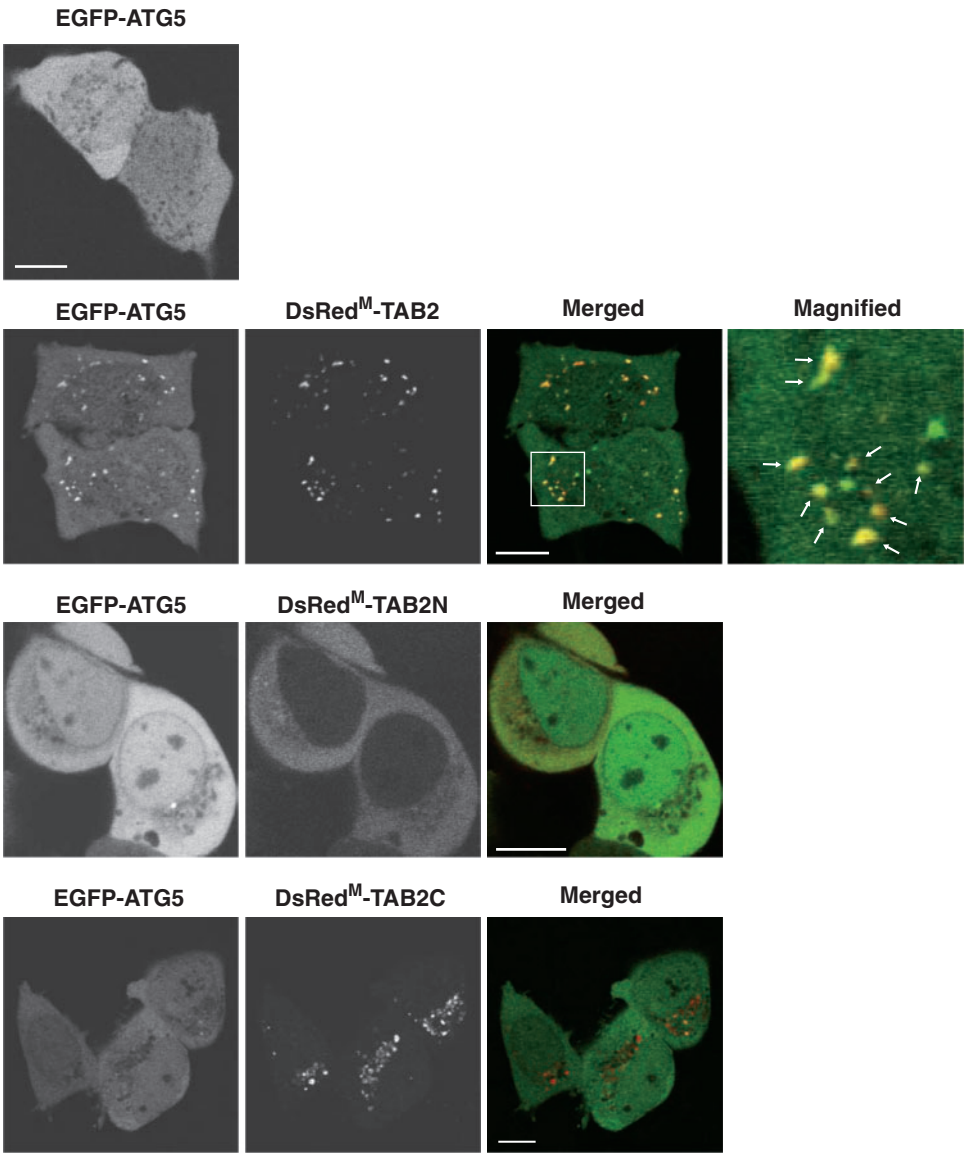

Fig. 3 TAB2 alters subcellular localization of ATG5. MCF-7 cells were transiently transfected with EGFP-ATG5 together with the DsRed^{M}-fused full-length TAB2, TAB2N or TAB2C as indicated. After transfection, cells were cultured in DMEM for 24 h and fixed using 4% paraformaldehyde. Fluorescent images were obtained with Carl Zeiss LSM510 confocal microscope. A square in the picture indicates the area shown in the magnified view. Arrows indicate colocalization of ATG5 and TAB2. The pinholes were set to capture an optical slice of ~1.0 μm. Scale bar = 10 μm.

TAB2 resulted in increase in the amount of p62/SQSTM1 under the normal culture condition, suggesting that overexpression of TAB2 can inhibit basal autophagy (Fig. 5A). Consistent with the subcellular localization of ATG5 (Fig. 3), the binding to Beclin1 or TAK1 seems to be required but not sufficient for this anti-autophagic function of TAB2, because both TAB2N and TAB2C did not affect the level of p62/SQSTM1 (Fig. 5A). We further examined whether the inhibition of basal autophagy by TAB2 depends on TAK1 activity by cotransfection of either wild-type or kinase negative mutant TAK1, and found that coexpression of TAK1 had no effect on TAB2-mediated autophagy inhibition regardless of TAK1 activity (Fig. 5A).

Recently, it was reported that TAB2 has dual roles in TAK1 regulation. TAB2 not only activates TAK1, but is also essential for deactivation of TAK1 upon certain stimuli such as TNF-α (*35*). We then investigated whether the autophagy induced by loss of TAB2 and TAB3 may be due to hyperactivation of TAK1. As shown in Fig. 5B, double knockdown of TAB2 and TAB3 resulted in decrease in the amount of p62/SQSTM1 as well as increase in the ratio of LC3-II/LC3-I in both wild-type and TAK1-deficient MEFs. Taken together, these data suggest that TAB2 and TAB3 negatively regulate basal autophagy independently of the kinase activity of TAK1.

TAB2 also interacts with ATG13 and is phosphorylated by ULK1

Two kinase complexes, the VPS34/Beclin1 complex and the ULK1 complex, are known to play essential roles in the early process of autophagosome formation.

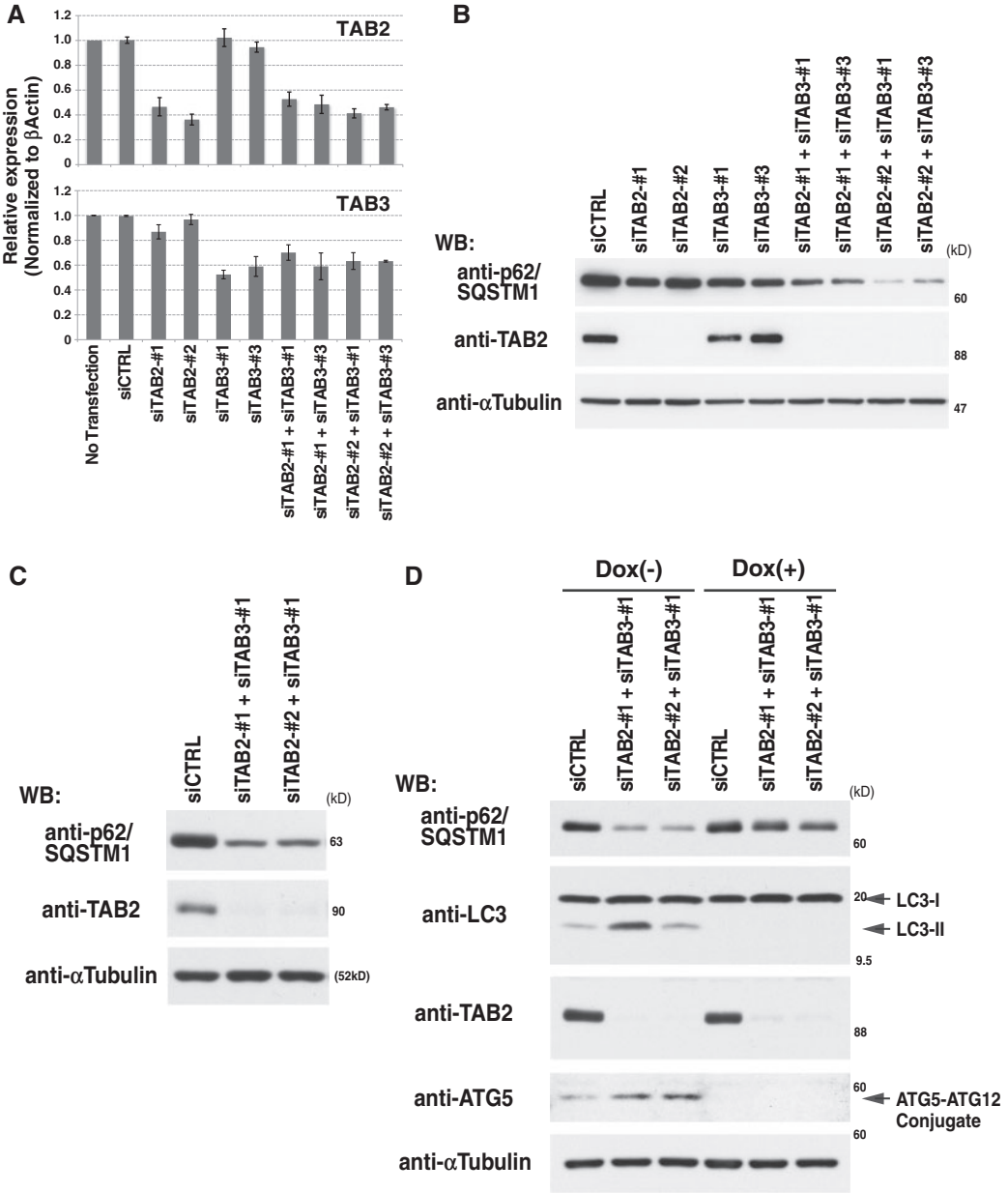

Fig. 4 Endogenous TAB2 and TAB3 suppress basal autophagy activity. (A) m5-7 cells were transfected with control siRNA (siCTRL) or siRNA against TAB2 and/or TAB3 (siTAB2 or siTAB3) as indicated and cultured in DMEM for 48 h. Total RNA was isolated and the relative expression levels of TAB2 and TAB3 compared to no transfection samples were determined by quantitative RT-PCR. Data were normalized to the level of βActin expression and are expressed as mean ± SEM of three independent experiments. (B) m5-7 cells were transfected with the indicated siRNAs and cultured in DMEM for 40 h. Whole-cell extracts were prepared and subjected to SDS–PAGE followed by western blotting (WB) using indicated antibodies. The amount of α-tubulin was monitored as a loading control. (C) MCF-7 cells were transfected with the indicated siRNAs and cultured in DMEM for 48 h. Whole-cell extracts were prepared and analysed by western blotting as in *B*. (D) m5-7 cells were cultured in the absence (−) or presence (+) of 1 μg/ml Doxycycline for two weeks prior to transfection. Then cells were transfected with the indicated siRNAs and cultured in DMEM for 50 h. Western blot analysis was performed using indicated antibodies.

Despite its essential role in autophagy regulation, the physiological substrate of ULK1 in this process has not been identified and a link between the ULK1 and the VPS34/Beclin1 complex remained unclear. Recently, Di Bartolomeo *et al.* reported that AMBRA1, one of the mammalian Beclin1-binding proteins (*36*), undergoes ULK1-dependent phosphorylation during autophagy induction and translocates to the ER (*37*). To gain insight into the mechanism by which TAB2 and TAB3 regulate autophagy, we examined the relationship between TAB2/TAB3 and the ULK1 complex. As shown in Fig. 6A, among the components of ULK1 complex tested, ATG13 was found to interact with both TAB2 and TAB3 (Fig. 6A and B). TAB2 was faintly detected in the anti-FLAG immunocomplexes in the presence of either ULK1 or FIP200 to a similar or lower level as the vector control, indicating that there is little interaction of TAB2 with these proteins under this experimental condition. But, interestingly, we found that the mobility of TAB2 protein on SDS–PAGE was obviously retarded when wild-type ULK1 was coexpressed (Fig. 6A).

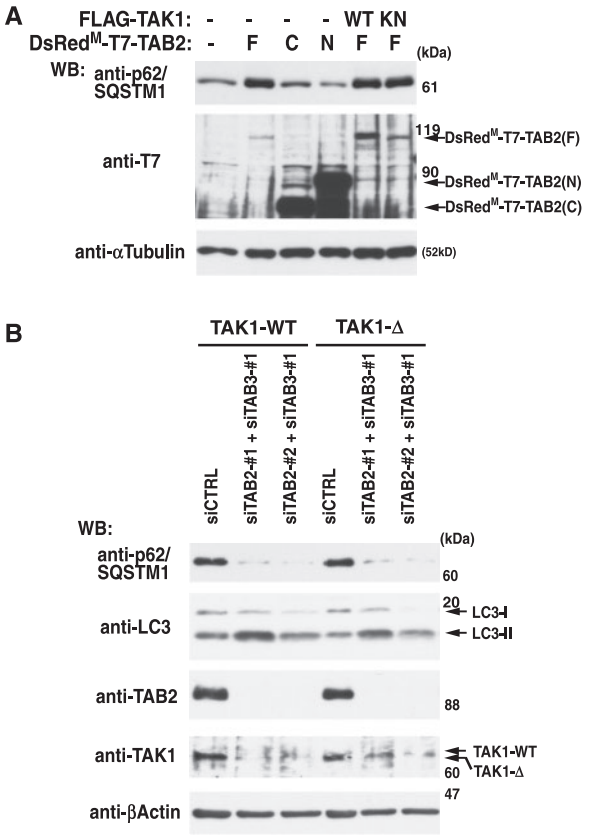

Fig. 5 TAB2 and TAB3 regulate basal autophagy independently of TAK1. (A) MCF-7 cells were transfected with expression plasmids for full length (F), the C-terminal (C) or the N-terminal (N) portion of TAB2 together with empty vector (−) or FLAG-TAK1 (WT, wild-type; KN, kinase negative mutant) as indicated and cultured in DMEM for 24 h. Whole-cell extracts were prepared and subjected to SDS–PAGE followed by western blotting using indicated antibodies. The amount of α-tubulin was monitored as a loading control. (B) Wild-type (TAK1-WT) or TAK1-deficient (TAK1-Δ) MEFs were transfected with control siRNA (siCTRL) or siRNAs against TAB2 and TAB3 (siTAB2 and siTAB3) as indicated and cultured in DMEM for 48 h. Whole-cell extracts were prepared and western blot analysis was performed using indicated antibodies. Note that TAK1-Δ is a truncated form of TAK1 in which exon 2 including ATP-binding site had been excised by Cre recombinase and therefore not functional as a kinase (*20*).

To determine whether TAB2 is phosphorylated by ULK1 *in vivo*, we employed a recently developed technique for detecting phosphorylated proteins, called Phos-tag. As shown in Fig. 6C, phosphorylation of TAB2 was detected when it is coexpressed with wild-type but not with kinase negative mutant ULK1, suggesting that TAB2 is phosphorylated *in vivo* in an ULK1-dependent manner. We could not detect phosphorylation of TAB3 by ULK1, but found that TAB3 can interact with ULK1 independently of its kinase activity (Fig. 6C). These results imply that TAB2 and TAB3 may provide a potential link between the two kinase complexes engaged in the autophagy regulation in mammalian cells.

Discussion

In this study, we have demonstrated a novel function for TAB2 and TAB3 as negative regulators of autophagy in mammalian cells. This function of TAB2 and TAB3 is likely to be independent of TAK1 activity, because overexpression of TAK1 (regardless of the wild-type or kinase negative mutant was used) did not affect the suppression of basal autophagy by TAB2 overexpression (Fig. 5A), and double knockdown of TAB2 and TAB3 enhanced basal autophagy in TAK1-deficient MEFs at a comparable level as observed in wild-type MEFs (Fig. 5B). Recently, Herrero-Martin et al. reported that TAK1 and TAB2 are required for TNF-related apoptosis-inducing ligand (TRAIL)-induced autophagy, protecting cells from apoptosis (*38*). These authors tested the single knockdown of either TAK1 or TAB2 and observed marginal effect on basal autophagy under normal culture condition. Together with these findings, our results suggest that TAB2 and TAB3 have an essential and overlapping role in the regulation of basal autophagy, while TAK1, at least as a protein kinase, does not seem to play such a role.

It is currently unclear how TAB2 and TAB3 regulate autophagy. Since overexpression of TAB2 induced the punctate localization of ATG5 and TAB2 itself colocalized with ATG5 as well as with Beclin1, it is possible that overexpressed TAB2 (and presumably TAB3 as well) might inhibit the process of autophagosome maturation by forming a non-functional initiation complex. The fact that TAB2C, which can still bind to Beclin1, does not induce the punctate localization of ATG5 (Fig. 3) or the accumulation of p62/SQSTM1 (Fig. 5A), suggests that the binding to Beclin1 is not sufficient for TAB2 to inhibit autophagy. There must be additional mechanism(s) for TAB2-mediated autophagy regulation, which involves the N-terminal portions of TAB2 and/or TAB3.

Our data indicate the redundant role of TAB2 and TAB3 in autophagy regulation. However, we also observed some difference between these two proteins. In the 293T overexpression system, TAB3 but not TAB2 was found to interact with ULK1, whereas TAB2 but not TAB3 was phosphorylated by ULK1 (Fig. 6). Because TAB2 and TAB3 can form homo- and hetero-oligomers at the endogenous level as well as under the overexpression condition (*17*), the ULK1 complex may interact with the TAB2/TAB3 heterooligomer through TAB3 and phosphorylate TAB2 at the endogenous level.

To date, there are several proteins reported as substrates of ULK1, which includes ATG13, FIP200, AMPK and AMBRA1 (*9*, *10*, *37*, *39*, *40*). AMBRA1, which is also one of the Beclin1-binding proteins, is phosphorylated by ULK1 upon starvation and promotes autophagy by recruiting Beclin1/VPS34 complex to the ER, where autophagosomes are nucleated (*37*). Our findings also raise the possibility that TAB2/TAB3 may function downstream of the ULK1 complex and upstream of the Beclin1/VPS34 complex in proximity to the site of autophagosome formation.

There seem to be at least two different pools of endogenous Beclin1 protein, one that localize to the trans-Golgi network (*41*), and the other that relocalize from cytoplasmic dynein motor complex to ER upon autophagy induction (*37*). Although the subcellular

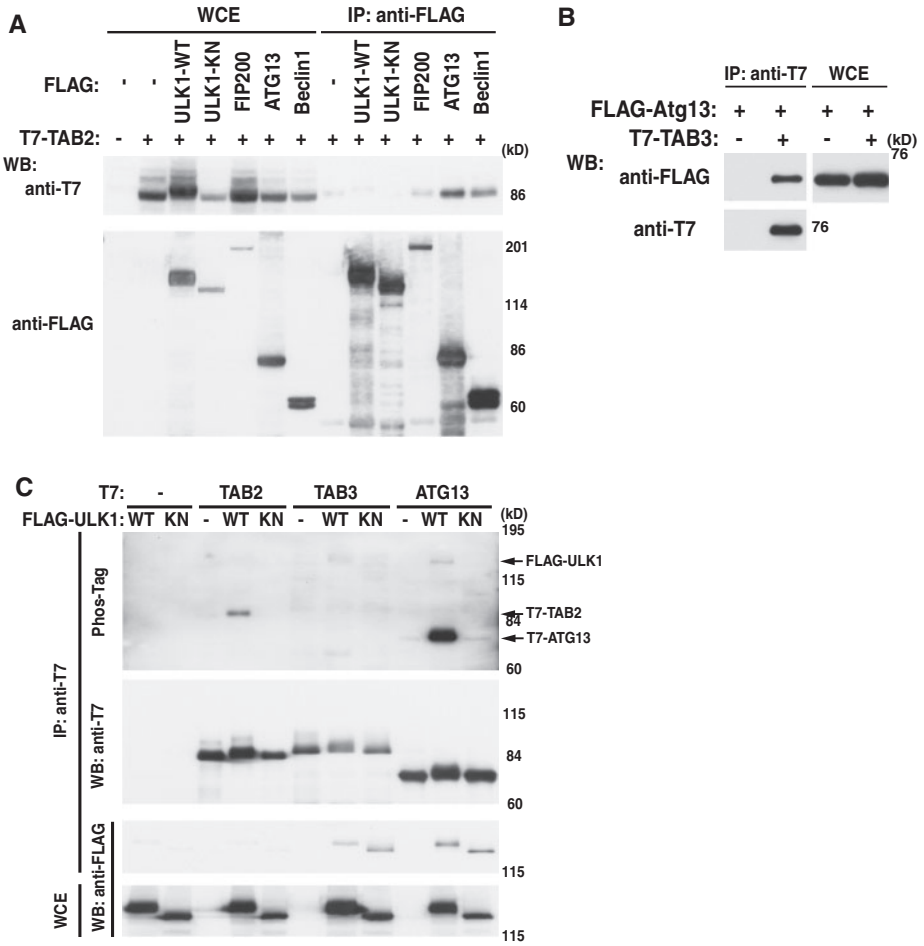

Fig. 6 TAB2 and TAB3 also interact with ULK1 complex. (A) 293T cells were transiently transfected with T7-TAB2 along with FLAG-tagged ULK1 (WT, wild-type; KN, kinase negative), FIP200, ATG13 or Beclin1 as indicated. Cell extracts were prepared at 24 h post-transfection and immunoprecipitated with anti-T7 antibody (IP), and analysed by western blotting with indicated antibodies (WB). Whole-cell extracts (WCE) were western blotted to monitor the amount of protein in the extracts. (B) 293T cells were transiently transfected with FLAG-Atg13 with (+) or without (−) T7-TAB3. Cell extracts were prepared at 24 h post-transfection and co-IP experiment was performed as indicated. (C) 293T cells were transiently transfected with T7-TAB2, -TAB3 or -ATG13 along with FLAG-tagged ULK1 (WT, wild-type; KN, kinase negative) or empty vector as indicated. Cell extracts were prepared at 24 h post-transfection and immunoprecipitated with anti-T7 antibody (IP). A portion of the immunoprecipitated complex was resolved by SDS–PAGE and transferred onto PVDF. The membrane was incubated with biotin-conjugated Phos-tag (Phos-tag BTL) and streptavidin-HRP to detect phospho-proteins. The immunocomplex (IP) and Whole-cell extracts (WCE) were also analysed by western blotting with indicated antibodies (WB).

localization of endogenous TAB2 and TAB3 still remains unclear, we speculate that endogenous TAB2 and TAB3 may localize in a different compartment from Beclin1 under the normal condition, and the phosphorylation of TAB2 by ULK1 may alter the subcellular localization and/or the protein structure of TAB2 that allows the interaction with Beclin1. In addition, it is even possible that the phosphorylation of TAB2 may switch the function of TAB2/TAB3 complex from autophagy-inhibitory to autophagy-stimulatory during autophagy induction, as the kinase activity of ULK1 is principally involved in the promotion of autophagy (*7*, *8*). Future investigation should determine the phosphorylation site(s) of TAB2 by ULK1 and its functional significance, such as the effects on autophagy activity and Beclin1 binding/localization.

The question still remains whether TAB2 and TAB3 only regulate the basal/constitutive autophagy or they also regulate the induced autophagy. To find a clue for the role of TAB2 and TAB3 in the induced autophagy, we have tested the interaction of endogenous TAB2 and Beclin1 under several autophagy-inducing conditions including nutrient starvation, treatment with TRAIL, etoposide, or lipopolysaccharide. However, so far we have been unable to detect the interaction of endogenous TAB2 with Beclin1 (data not shown). Thus, we speculate that TAB2 and TAB3 may not be involved in autophagy regulation in general, but rather involved in autophagy regulation in specific situation that we have not yet examined, such as selective autophagy for the elimination of protein aggregates, damaged organelles or intracellular pathogens. Further studies are needed to clarify the physiological relevance and the detailed molecular mechanism by which TAB2 and TAB3 regulate autophagy.

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Conflict of interest
None declared.

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