# TRAF6 activation of PI 3-kinase-dependent cytoskeletal changes is cooperative with Ras and is mediated by an interaction with cytoplasmic Src

**Kent Z. Q. Wang[1], Nawarat Wara-Aswapati[2], Jason A. Boch[3], Yasuhiro Yoshida[4], Chang-Deng Hu[5], Deborah L. Galson[1,6] and Philip E. Auron[1,*]**

[1]Department of Molecular Genetics and Biochemistry, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA
[2]Department of Periodontology, Faculty of Dentistry, Khon Kaen University, Khon Kaen, 40002, Thailand
[3]Department of Medicine, Harvard Medical School and Beth Israel Deaconess Medical Center, Boston, MA 02115, USA
[4]Department of Immunology, University of Occupational and Environmental Health, Kitakyushu, 807, Japan
[5]Department of Medicinal Chemistry and Molecular Pharmacology, Purdue University School of Pharmacy, West Lafayette, IN 47907, USA
[6]Center for Bone Biology, Department of Medicine, University of Pittsburgh, Pittsburgh, PA 15261, USA
*Author for correspondence (e-mail: auron@pitt.edu)

*Accepted 9 January 2006*
*Journal of Cell Science 119, 1579-1591 Published by The Company of Biologists 2006*
*doi:10.1242/jcs.02889*

## Summary

**Interleukin 1 (IL-1) has been implicated in the reorganization of the actin cytoskeleton. An expression vector encoding a PKB/Akt pleckstrin-homology domain fused to a fluorescent protein was used to detect phosphoinositide 3-kinase (PI 3-kinase) products. It was observed that PI 3-kinase was activated either by treatment with IL-1 or by expression of either TRAF6, Src, MyD88 or dominant-positive PI 3-kinase, and resulted in the formation of long filopodia-like cellular protrusions that appeared to branch at membrane sites consisting of clusters of phosphoinositide. This depended upon a TRAF6 polyproline motif and Src catalytic activity, and was blocked by inhibitors of PI 3-kinase, Src and Ras. Using both conventional and split fluorescent protein probes fused to expressed TRAF6 and Src in living cells, the polyproline sequence of TRAF6 and the Src-homology 3 (SH3) domain of Src were shown to be required for interaction between these two proteins. Interaction occurred within the cytoplasm, and not at either the cell membrane or cytoplasmic sequestosomes. In addition, co-transfection of vectors expressing fluorescent-protein-fused TRAF6 and non-fluorescent MyD88, IRAK1 and IRAK2 revealed an inverse correlation between increased sequestosome formation and activation of both PI 3-kinase and NF-κB. Although a key factor in TRAF6-dependent activation of PI 3-kinase, ectopic expression of Src was insufficient for NF-κB activation and, in contrast to NF-κB, was not inhibited by IRAK2.**

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/119/8/1579/DC1

Key words: Filopodia, BiFC/Split GFP, Sequestosome, Proteasome, IL-1/Toll receptor

## Introduction

The tumor necrosis factor receptor (TNFR)-associated factor (TRAF) cytoplasmic signal transducers mediate many cellular activities for both the TNFR and the TIR [for 'Toll-interleukin (IL)-1/18 receptor] families. The TIR family is characterized by a common, homologous cytoplasmic TIR domain. Of at least 26 TNFR molecules, 23 transduce signals through one or more TRAF molecules (Locksley et al., 2001). Of these, 14 directly bind TRAF to cytoplasmic TRAF-interaction motifs (TIMs). Some TNFRs, such as TNFR1, recruit a TRAF indirectly through an adaptor molecule such as TRADD, which engages the receptor cytoplasmic sequence through a homotypic death-domain interaction (Dempsey et al., 2003). Like TNFR1, TIR transmembrane receptors do not directly recruit TRAF, but utilize a two-tier adaptor mechanism involving association with cytoplasmic TIR-domain-containing molecules including MyD88, TIRAP/Mal and TRIF which, in turn, utilize a distinct death-domain interaction to recruit members of the IL-1 receptor-associated kinase (IRAK) family (Dunne and O'Neill, 2003). The IRAK molecules carry multiple TIM sequences that exclusively bind to TRAF6 (Ye et al., 2002). Although TNFRs can collectively activate all agonistic TRAF isoforms, some receptors possess defined specificities for TRAF6 versus other TRAFs. By contrast, TIRs exclusively activate TRAF6 (Akira and Takeda, 2004). TNFR, like CD40 and RANK, have been shown to recruit TRAF molecules directly to the cell membrane (Hostager et al., 2000) and to associate into methyl-β cyclodextrin (MbCD)-sensitive membrane microdomains (Brown et al., 2001; Ha et al., 2003; Vidalain et al., 2000).

IRAK can be recruited to TIR molecules at the cell membrane by MyD88; however, in contrast to the TNF-family receptor RANK (Wong et al., 1999), TRAF6 has not been reported to be recruited by TIR to the membrane. This suggests that, following activation by a TIR, IRAK binds to the receptor and is either subsequently released to interact with TRAF6 in the cytoplasm or TRAF6 interacts with, and rapidly releases, receptor-bound IRAK. Consequently, such a transient

interaction might not be readily captured by the experimental techniques used. Interestingly, overexpression of TRAF6 functions as a dominant positive; however, when overexpressed, green fluorescent protein (GFP)-TRAF6 appears to be localized both to the general cytoplasm and to punctate cytoplasmic structures (Force et al., 2000; Zapata et al., 2001) called sequestosomes (Seibenhener et al., 2004), especially following exposure to a TIR ligand such as IL-1 (Sanz et al., 2000). Five agonistic TRAF isoforms are believed to function as homo- or heterotrimers, possessing an N-terminal Ring and zinc finger (RZF) region, a central coiled-coil (CC) region, and a C-terminal Ig-like meprin and TRAF homology (MATH) domain. The RZF region has been reported to be crucial for downstream activation of MAP kinases (MAPKs) and NF-κB (Baud et al., 1999), through interaction with kinases such as MEKK1, TAK1 and atypical protein kinase C (PKC)ζ and λ/ι (Duran et al., 2004; Sanz et al., 2000). The CC region mediates both multimerization and the recruitment of the E2 ubiquitin conjugating complex Ubc13/Uev1A, which is crucial for the non-degradative K63 polyubiquitylation that is important for TRAF activity (Yang et al., 2004). The MATH domain might contribute to multimerization, but also contains a groove that specifically binds distinct TIMs that link TRAF to upstream receptor signaling (Ye et al., 2002). TRAF6, in contrast to other family members, has been reported to activate several distinct signal pathways. For example, interaction with signaling proteins ECSIT (Kopp et al., 1999) or p62 (also known as ZIP and SQSTM1) (Layfield and Hocking, 2004; Moscat et al., 2001) facilitates the recruitment of kinases MEKK1 and atypical PKCζ, respectively (Sanz et al., 2000). These adaptors bind directly to the MATH-domain TIM groove and facilitate recruitment of the relevant kinase to the RZF region. An additional TRAF6-specific pathway involves activation of both Src and phosphoinositide 3-kinase (PI 3-kinase). This unique pathway appears to rely upon Src interaction with TRAF6, and has been reported to be dependent, in vitro, upon the polyproline (polyPro) sequence located exclusively within the MATH domain of TRAF6 (Wong et al., 1999).

The importance of PI 3-kinase activation to processes involving alterations in cell morphology underscores a crucial function that is also associated with the action of TNFR and TIR receptors. For example, IL-1 treatment of cells has been reported to initiate rapid changes in cell shape (Puls et al., 1999) that depend upon the catalytic function of PI 3-kinase (Lee et al., 2004b). This has especially been noted for osteoclast differentiation where IL-1, lipopolysaccharide and the TNF-like molecule RANK ligand activate osteoclast differentiation in a manner that depends upon TRAF6 and PI 3-kinase (Kobayashi et al., 2001; Wong et al., 1999). Src (reviewed by Gravallese et al., 2001) and, to a lesser extent, the mono-acylated form of Hck (Jeschke et al., 1998; Lowell et al., 1996) have been implicated in the process of osteoclast activation and/or differentiation. However, the exact role and importance of the kinase catalytic domain has been debated (Xing et al., 2001). Since TRAF-family molecules are the primary mediators of TNF and IL-1 signaling, it has been assumed that overexpression of TRAF6, which provides a dominant-positive signal for MAPK and NF-κB activation, should similarly initiate a TNF-like and/or IL-1-like alteration in cell morphology. To date, the only report of such an attempt, involving protein microinjection of a TRAF6 expression vector into Swiss 3T3 cells, has revealed a lack of such function (Puls et al., 1999).

We report here that overexpression of TRAF6 in transfected HEK293 cells activates PI 3-kinase activity and a program of actin polymerization that results in the growth of long filopodia. These filopodia appear to emanate from clusters of phosphoinositide that are reminiscent of those initiated by Wiskott-Aldrich syndrome protein (WASP)-activated Arp2/3 complex (Millard et al., 2004). Similar responses can be generated by dominant-positive PI 3-kinase, Src, MyD88 or treatment with IL-1. The TRAF6-dependent activation is inhibited by inhibitors of PI 3-kinase, Src and Ras, and appears to be dependent upon the integrity of the Src kinase catalytic domain. Using both conventional and bimolecular fluorescence complementation (BiFC) probes, we investigated the role of the MATH-domain polyPro sequence in TRAF6 signaling. Our results argue for TRAF6 interaction with the Src-homology 3 (SH3) domain of Src within the cell cytoplasm, and not at either the cell membrane or the previously described cytoplasmic structures called sequestosomes (Layfield and Hocking, 2004; Seibenhener et al., 2004). Finally, we observed that overexpression of either MyD88 or Src with yellow fluorescent protein (YFP)-labeled TRAF6 reduces sequestosomal localization of TRAF6 without affecting its ability to activate both NF-κB and PI 3-kinase.

## Results

### IL-1 activates cellular morphology changes through TRAF6 and PI 3-kinase

IL-1 has been reported to generate cellular morphological changes by reorganization of the actin cytoskeleton (Puls et al., 1999). However, details of the mechanism are unclear. Because of its relevance to cytoskeletal rearrangement, we set out to examine the mechanism of PI 3-kinase activation by IL-1 in living cells using a fluorescent probe that can detect the presence of PI 3-kinase-dependent phosphoinositides. This was accomplished by transfecting a cDNA expression vector encoding the specific phosphoinositide-binding PKB/Akt pleckstrin-homology (PH) domain fused to GFP (PH-Akt-GFP). Through changes in membrane fluorescence, this allows visualization in the cell membrane of stimulus-induced increases in phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)$P_3$] as well as phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)$P_2$] and phosphatidylinositol (3,4)-bisphosphate [PtdIns(3,4)$P_2$]. Hereafter, the two bisphosphate forms will be referred to collectively as PIP2. While investigating different cell lines, we observed that the activation of PI 3-kinase in response to IL-1 can be either rapid or slow, depending on the cell type. Following IL-1 treatment of Hep3B hepatocytes previously transfected with PH-Akt-GFP, there is a rapid (within 10 minutes) increased localization of PH-Akt-GFP to a region on the cell surface and into filopodia-like protuberances (Fig. 1A). By contrast, HEK293 cells [HEK293R (Yoshida et al., 2004)] stably transfected with a cDNA expression vector for the type I IL-1 receptor (IL-1$R_I$), require more than 4 hours of IL-1 treatment, exhibiting a strong response by 24 hours (Fig. 1B). Fig. 1C shows that relocalization of PH-Akt-GFP to the membrane can be substantially reversed by treatment with LY294002, a specific PI 3-kinase inhibitor.

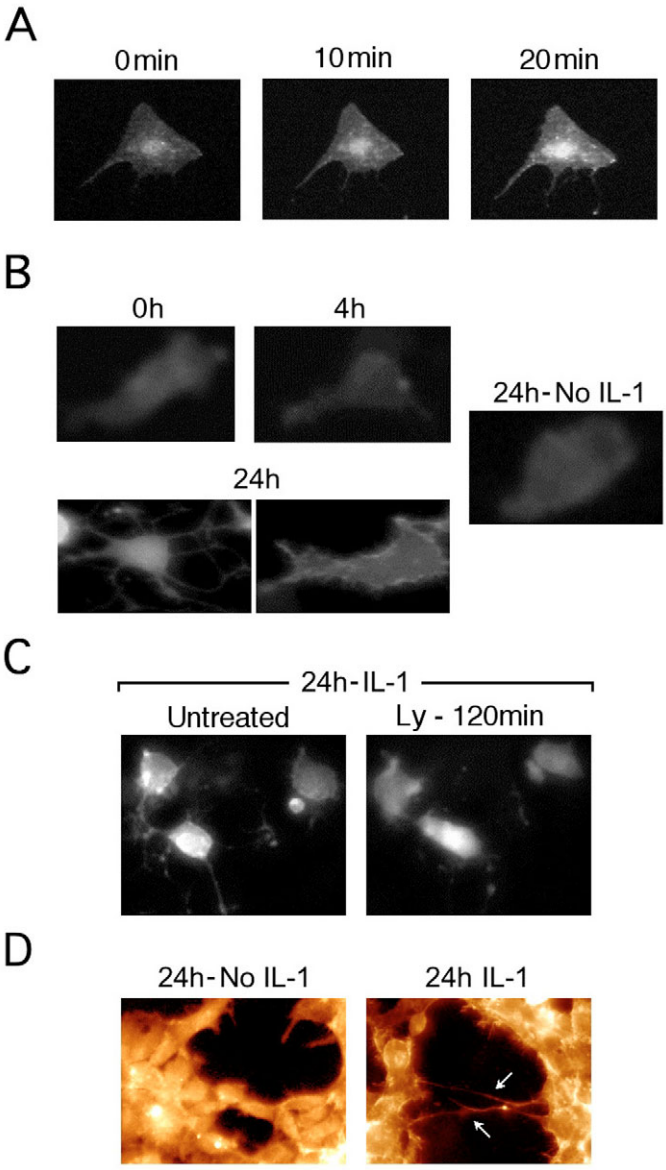



**Fig. 1.** IL-1β induces PI 3-kinase-dependent filopodia growth and translocation of PH-Akt-GFP. Hep3B (A) and HEK293R (B,C) cells were transfected with 15 ng of PH-Akt-GFP expression vector in a 96-well plate for 24 hours. IL-1β was then added, or not (24h-No IL-1), into each well at a final concentration of 10 ng/ml for the indicated time. (C) Following 24 hours of IL-1 treatment, cells transfected with PH-Akt-GFP were exposed to a final concentration of 25 μM LY294002 (Ly). (D) HEK293R cells were fixed and stained with phalloidin-TRITC following incubation for 24 hours either in the absence or presence of IL-1β, as indicated. Arrows locate long, branched filopodia-like structures.

In order to distinguish between PH-Akt-GFP recruitment to pre-existing filopodia-like structures versus de novo generation of filopodia, control and IL-1-treated cells were stained with phalloidin-TRITC, a specific fluorescent filamentous-actin-binding reagent. Fig. 1D shows that 24 hours of IL-1 treatment of HEK293R cells generates very long actin-containing filopodia, which are not observed in the control.

It has been reported that IL-1 cytoskeleton rearrangement in corneal endothelial cells is dependent upon PI 3-kinase (Lee et al., 2004a). Since IL-1R signal transduction depends upon downstream activation of TRAF6 (Cao et al., 1996), which has been demonstrated to function as a dominant positive when overexpressed (Medzhitov et al., 1998), we attempted to induce changes in cell morphology by TRAF6 overexpression. To investigate whether overexpression of TRAF6 in cells can activate both PI 3-kinase and cell morphological changes, expression vectors encoding TRAF6 and PH-Akt-GFP were co-transfected into IL-1$R_I$-deficient HEK293 cells. In the absence of ectopic TRAF6 expression, PH-Akt-GFP is primarily localized to the cytoplasm (Fig. 2A, a). However, when TRAF6 was overexpressed in these cells, filopodia-like protrusions were observed, and fluorescence shifted from the cytoplasm to cell membranes (Fig. 2A, b). A correlation between this observation and PI 3-kinase activation was confirmed by co-transfection of a dominant-positive PI 3-kinase construct encoding the p110 catalytic subunit with the PH-Akt-GFP vector in HEK293 cells, resulting in a similar response (Fig. 2A, c). This strategy was repeated in RAW264.7 cells, a monocyte-macrophage cell line that can be differentiated upon cytokine stimulation (Matsuo et al., 2004), again resulting in recruitment to membranes and protrusions (Fig. 2A, d-f). Overexpression of either TRAF6 or Src, another activator of cell morphological change, results in the presence of filopodia in the absence of PH-Akt-GFP following staining with phalloidin-TRITC (Fig. 2A, g-I, arrows). Therefore, TRAF6, like IL-1, appears to be able to induce formation of protrusions.

Incubation of cells with 25 μM LY294002, a PI 3-kinase inhibitor, 24 hours after TRAF6 and PH-Akt-GFP co-transfection resulted in the loss of cellular protrusions and a return of fluorescence to the cytoplasm within 60 minutes (Fig. 2B, a-c). The DMSO vehicle control did not reveal any visible change within 2 hours (Fig. 2B, j-l). To test whether the translocation of PH-Akt-GFP was dependent upon the formation of microdomains in the cell membrane, the cells co-transfected with TRAF6 and PH-Akt-GFP were treated with 10 mM MbCD, to potentially disrupt membrane microdomain-dependent signaling (Scheiffele et al., 1997). Like LY294002, MbCD reduced the fluorescent labeling of protrusions (Fig. 2B, d-f) and membranes (Fig. 2B, g-i), possibly owing to an effect on the membrane localization of PtdIns(3,4,5)$P_3$ and PIP2. It has been well documented that cellular protrusions such as filopodia and lamellipodia are formed by the polymerization of cytoplasmic G actin, and PI 3-kinase is a key positive regulator for these processes (Janmey and Lindberg, 2004; Revenu et al., 2004). We therefore stained the cells co-transfected with TRAF6 and PH-Akt-GFP with phalloidin-TRITC. As demonstrated in Fig. 3A, the PH-Akt-GFP signal (green) coincides with the filamentous actin (red) staining most strikingly at specific loci. Some of these loci correspond to bifurcation points, where one actin bundle appears to emanate from another at acute angles (Fig. 3A). Such angular bifurcations might reflect the existence of actin molecular junctions mediated by the Arp2/3 protein complex (Millard et al., 2004). Arp2/3 generates a 70° actin molecular branch that results in filopodia branching at acute angles. This branching depends upon the recruitment and activation of WASP-family factors to PtdIns(4,5)$P_2$, which is one type of phosphatidylinositol

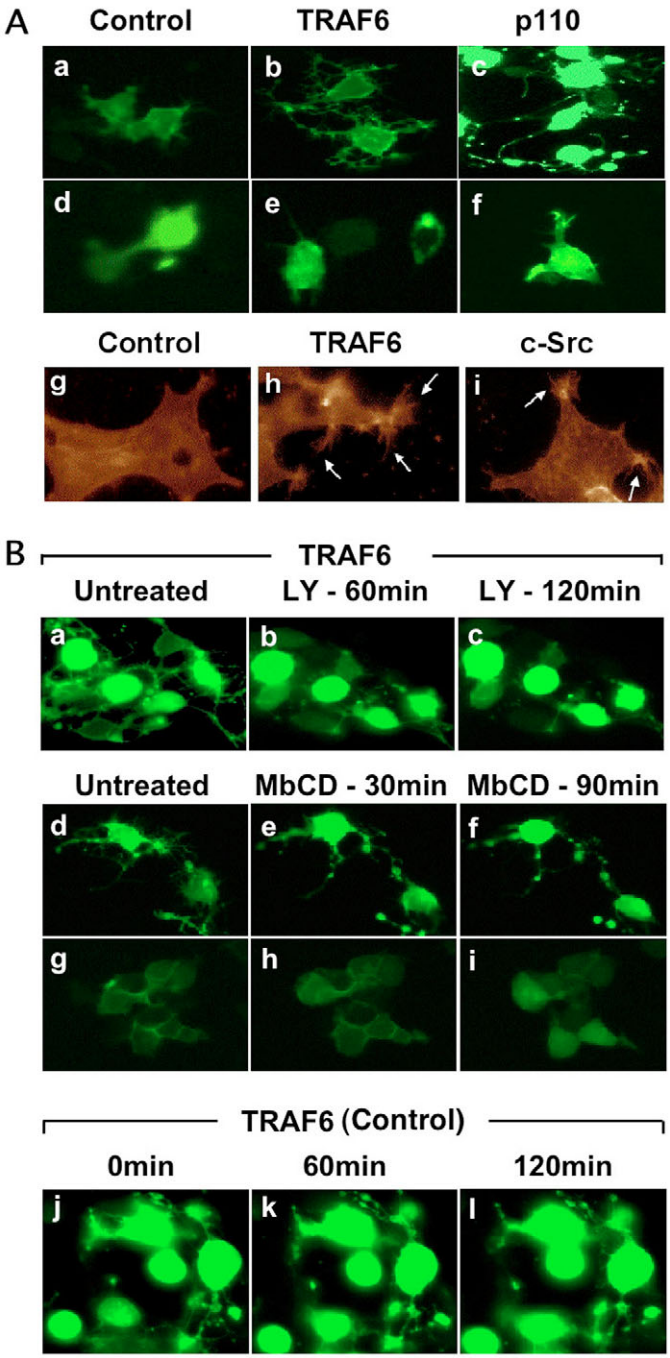



**Fig. 2.** TRAF6 induces translocation of PH-Akt-GFP to cell membranes and de novo protrusions. (A) A PH-Akt-GFP expression vector reporter was co-transfected with expression vectors encoding a second activator protein, as indicated above each panel, into either HEK293 (a-c) or RAW264.7 (d-f) cells for 24 hours. Transfection of only the activator protein, followed by fixation and phalloidin-TRITC staining in order to visualize polymerized actin (g-i). (B) PH-Akt-GFP expression vector reporter co-transfected with a TRAF6 expression vector into HEK293 cells in either the absence or presence of 25 μM of Ly294002 (LY) and 10 mM methyl-β-cyclodextrin (MbCD) inhibitors for the indicated treatment time (a-i). Two different sets of cells are shown for MbCD. In one (d-f), a long exposure time reveals the details of the filopodial network. In another (g-i), a shorter exposure reveals that the PH-Akt-GFP is relocalized from cell membranes to the cytoplasm, following treatment with MbCD. A control set shows the co-transfected vectors evaluated in the presence of the DMSO vehicle for an equivalent treatment time (j-l) with a long exposure time to allow visualization of filopodia.

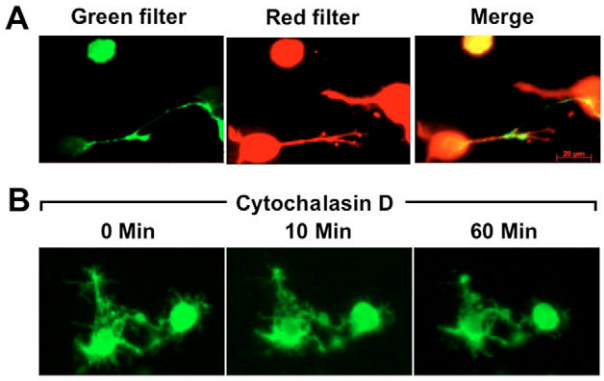



**Fig. 3.** TRAF6 induces the localization of PH-Akt-GFP to actin branch points. (A) HEK293 cells were co-transfected with a PH-Akt-GFP expression vector reporter and a TRAF6 expression vector for 24 hours, followed by fixation and phalloidin-TRITC staining. GFP (green), FITC (red) and merged images were used to determine signal co-localization (yellow). (B) HEK293 cells were co-transfected with PH-Akt-GFP and TRAF6 expression vectors for 24 hours, then treated for the indicated times with 5 μM cytochalasin D.

diphosphate, generated within the membrane by various pathways that indirectly depend upon PI 3-kinase. The pathway argued to be the most important depends upon phosphatidylinositol-4-phosphate 5-kinase, which is activated by PtdIns(3,4,5)$P_3$ membrane recruitment of two different mediators, Tec-family kinases (Saito et al., 2003) and Rho (Weernink et al., 2004). As expected, treatment at 24 hours post-transfection with a specific filamentous-actin-depolymerization reagent, cytochalasin D, resulted in a decrease in PH-Akt-GFP-containing protrusions (Fig. 3B). This further demonstrates that PH-Akt-GFP localizes into actin-containing structures.

### TRAF6 MATH-domain polyPro motif is important for the activation of PI 3-kinase and NF-κB

The TRAF6 molecule transduces many IL-1R signals by protein-protein interaction. The N-terminal RZF region in TRAF6 has been reported to function as a mediator of downstream events such as IκB kinase (IKK) and MAPK activation (Baud et al., 1999). However, the C-terminal MATH domain, which is often defined as a structure involved in the integration of upstream receptor-proximal signals, has also been reported to be involved in downstream signal transduction. Examples include RANK ligand signaling through its receptor in osteoclasts (Wong et al., 1999) and the super-activation of the NF-κB p65 homodimer by IL-1 (Yoshida et al., 2004). The involvement of the MATH domain in RANK signaling has been reported to depend upon the integrity of a proline-rich putative SH3-domain-binding motif (PxxPxxP) that is exclusively found in TRAF6, located between amino acids 462-468. This sequence was reported to interact physically with Src tyrosine kinase, which activates PI

3-kinase (Wong et al., 1999). However, the polyPro interaction in this report was specifically characterized only by in vitro protein-protein association as a triple Pro-to-Ala substitution. This is a concern not only because of the lack of in vivo analysis, but also because comparison of the structures of the isolated TRAF6 MATH-domain monomer (Ye et al., 2002) with the more-complete TRAF2 CC region plus MATH-domain trimer (Park et al., 1999) predicts that Pro462, which unlike the two other prolines is conserved in all TRAF-family members, should be buried near the MATH interdomain interaction surface (see molecular modeling data: Fig. S1, supplementary material). Consequently, the triple mutation could have compromised the structural integrity of the TRAF trimer, resulting in a nonspecific loss of interaction.

To verify the specificity of the polyPro sequence as a functional component of TRAF6, we constructed expression vectors encoding wild-type and three polyPro sequence substitution variant proteins (Fig. 4A). A triple Pro-to-Ala substitution (P3A), similar to that previously reported (Wong et al., 1999), a double substitution P465A and P468A (P2A), as well as a more-specific P465A, were designed to eliminate the Src SH3 interaction of the exposed PxxP loop between residues 465 and 468 without involving P462, which might be crucial for structure. When these plasmids were co-transfected with PH-Akt-GFP into HEK293 cells, we found that none of the polyPro substituted variants was capable of activating PI 3-kinase, as compared with the wild-type TRAF6 (Fig. 5A). When the variant proteins were assayed for NF-κB activity using an NF-κB luciferase reporter specific for p50/p65 heterodimers (Yoshida et al., 2004), P465A and P2A revealed reduced activity, whereas P3A was inactive (Fig. 5B). The complete loss of activity by TRAF6-P3A might be due to the predicted effect on structural integrity and/or multimerization, as hypothesized above. It is unlikely that the loss of both PI 3-kinase and NF-κB activities is a result of a lack of expression for the TRAF6-P3A, since these same expression vectors, which generate YFP-fused forms of the TRAF6 proteins, all reveal similar amounts of expression and patterns of cellular localization (Fig. 6D) distinct from that of non-fused YFP (Fig. 6C, e). However, since TRAF6-P465A and P2A revealed significant, although reduced, NF-κB activity (Fig. 5B), the reduction compared with wild-type TRAF6 might be the result of a partial effect on structural integrity.

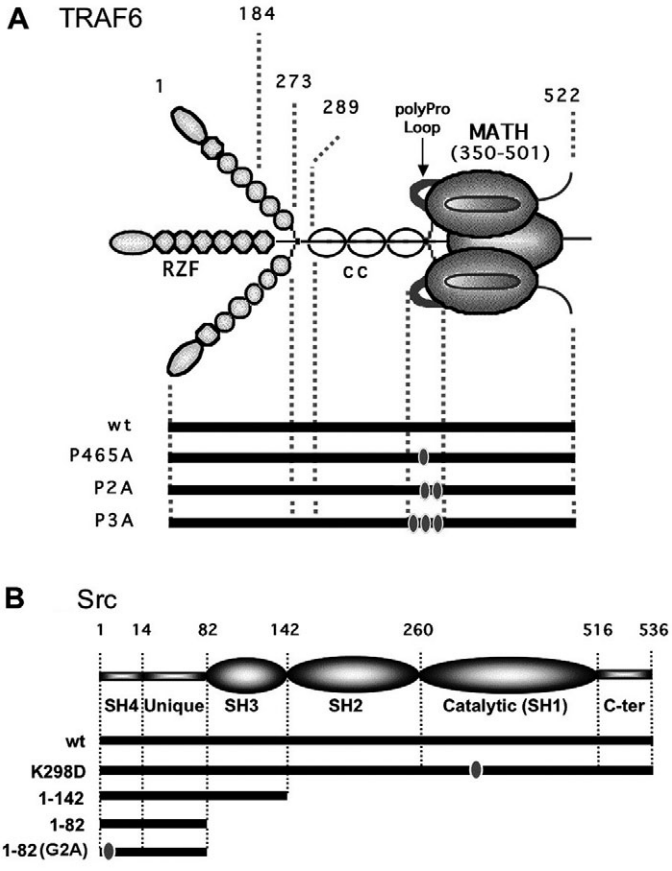



**Fig. 4.** TRAF6 and Src proteins used in these studies, showing the relative location of structural-functional domains. (A) Schematic of wild-type (wt) TRAF6 trimer structure and the location of point mutations. (B) Wild-type human Src and the location of point and deletion mutations.

## Visualizing a TRAF6-Src interaction in living cells

As described above, previously reported in vitro studies have shown that TRAF6 directly binds to Src. However, direct evidence for the interaction between TRAF6 and Src has not been demonstrated in living cells. Although our observation that substitution of crucial proline residues results in a loss of TRAF6 dominant-positive activity is consistent with the previously proposed Src SH3 domain interaction, it does not provide direct evidence for association in living cells. As an approach to visualizing such an interaction directly, the C-

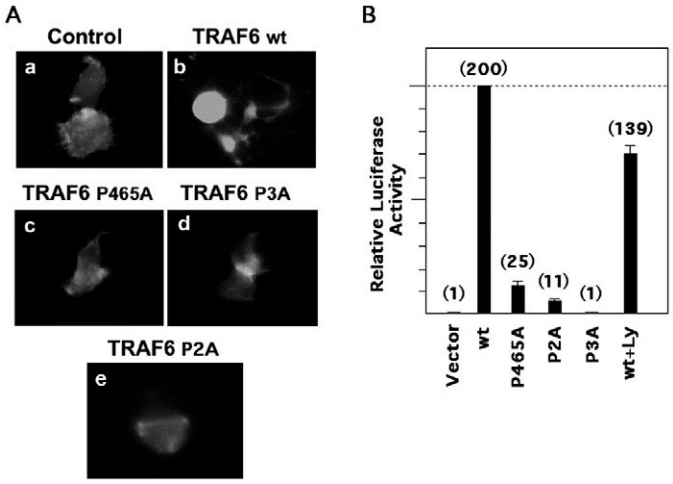



**Fig. 5.** Prolines within the TRAF6 MATH domain are required for morphological changes and activation of both PI 3-kinase and NF-κB. (A) HEK293 cells were co-transfected with a PH-Akt-GFP expression vector reporter and various TRAF6 constructs (as described in Fig. 4A) for 24 hours. (B) An NF-κB-dependent luciferase reporter containing four tandem κB-binding sites adjacent to a minimal *fos* promoter was assayed 24 hours following cell transfection with either 150 ng of various TRAF6 expression vectors or control vector into HEK293 cells as described in the Materials and Methods. The PI 3-kinase inhibitor LY294002 (Ly) was added at a final concentration of 25 μM into cells after 2 hours of transfection and maintained until analyzed for luciferase activity. Numbers in parentheses indicate fold activity over control vector.

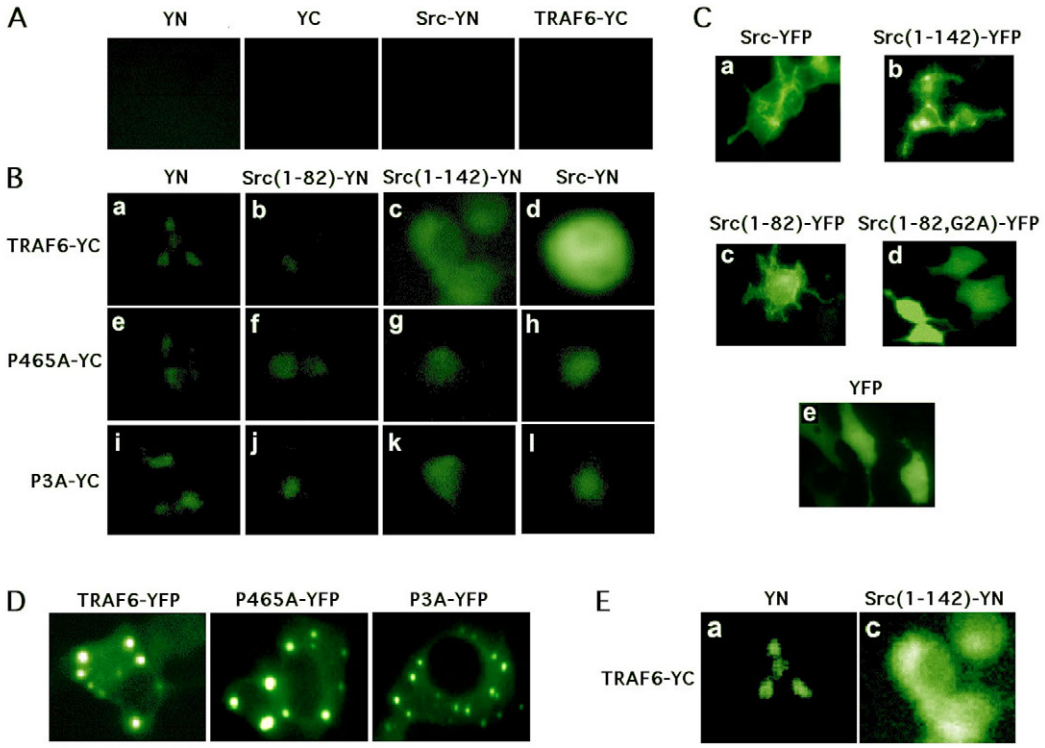



**Fig. 6.** Examination of subcellular localization and protein-protein interaction in living cells for de novo-expressed Src and TRAF6. Bimolecular fluorescence complementation (BiFC) involving split YFP tags was used to detect intracellular protein-protein interaction between TRAF6 and Src that could be compared with the expression pattern for these proteins tagged with full-length YFP in HEK293T cells transiently transfected for 24 hours. (A) Transfection of 200 ng of individual, uncomplemented BiFC vectors; –YN, N-terminal YFP fragment (aa 1-154), –YC, C-terminal YFP fragment (aa 155-238). (B) Transfection of 200 ng of each BiFC vector complementary pair (–YN + –YC). (C) Subcellular localization of Src-YFP and variants in cells transfected with 50 ng of expression vectors encoding each protein form, as indicated. (D) Subcellular localization of TRAF6-YFP and variants in cells transfected with 20 ng of expression vectors as indicated. (E) Image enhancement of Fig. 6B panels a and c, revealing distinct TRAF6 and Src morphologies.

termini of wild-type, P465A and P3A TRAF6 cDNA were each ligated to cDNA encoding both full-length YFP and to a C-terminal YFP fragment (–YC: aa 155-238) that has been demonstrated to complement a second N-terminal YFP fragment (–YN: aa 1-154) and generate a fluorescent signal (Hu et al., 2002a). This technique, which has been referred to as BiFC, is a variant of a protein complementation assay approach that provides direct visualization of protein-protein interactions in living cells with a resolution on the order of 10 Å (Michnick, 2001).

We ligated the N-terminal YFP BiFC cDNA to the C-terminus of various Src cDNA sequences. Transfection of expression vectors encoding only one part of the YFP BiFC pair (–YN or –YC) into HEK293T cells did not yield any detectable fluorescence (Fig. 6A). When TRAF6-YC was co-transfected with an expression vector encoding only the YN-BiFC complement, a very weak punctate signal was observed (Fig. 6B and Fig. 6E, a). The punctate nature of the signal resembles the punctate subcellular localization observed for YFP-tagged TRAF6 (Fig. 6D) that has also been reported by several other groups for other TRAF-family molecules (Force et al., 2000; Zapata et al., 2001). The punctate structures have been designated as sequestosomes, the site of TRAF6 interaction with the PKCζ-interacting protein p62 (Seibenhener et al., 2004). A similar weak punctate pattern is also observed for TRAF6-YC co-transfected with Src (1-82)-YN (Fig. 6B, b), a short N-terminal construct missing the SH3 domain (Fig. 4B). These weak signals probably represent a low level of association for the complementary split YFP peptides in the absence of secondary protein-protein interaction domains. By contrast, when either full-length Src-YN (Fig. 6B, d) or Src-(1-142)-YN (Fig. 6B, c), encoding the N-terminus containing the SH3 domain (Fig. 4B), were co-transfected with TRAF6-YC, a stronger fluorescent signal was observed to be spread more uniformly throughout the cytoplasm.

These results are consistent with a dependence upon the SH3 domain for TRAF6 interaction with Src. However, the greater intensity of the BiFC signal for full-length Src versus Src-(1-142) suggests that additional interactions might be involved. Conventional YFP-tagged forms of various Src peptides have revealed similar expression levels (Fig. 6C). The shift in subcellular localization from punctate structures to a more uniform cytoplasmic distribution is especially obvious when the images shown in Fig. 6B panels a and c are enhanced by digital contrast adjustment (Fig. 6E). This suggests that the TRAF6-Src interaction does not occur within the punctate structures. It should also be emphasized that the BiFC interaction pattern does not resemble that of the various YFP-Src constructs containing the SH4 myristoylation signal, which results in both cytoplasmic and membrane localization (Fig.

6C, a-c). However, it does resemble the result obtained when the crucial position 2 glycine myristoylation signal is substituted, resulting in an exclusively cytoplasmic localization (Fig. 6C, d). Therefore, the TRAF6-Src interaction does not appear to occur at the membrane, but rather is a predominantly cytoplasmic event. Finally, TRAF6 polyPro substitution variants P3A-YC and P465A-YC co-transfected with the various Src-YN constructs all displayed a greatly reduced signal (Fig. 6B, e-l). Since the TRAF6 polyPro variants all appeared to be expressed at levels similar to wild-type TRAF6 (Fig. 6D), the decreased BiFC signal argues that interaction between TRAF6 and Src depends primarily upon the integrity of the polyPro sequence. However, as suggested by the difference between the BiFC signals for Src and Src-(1-142), the efficiency of the interaction might depend upon additional Src sequences carboxyl to the SH3 domain.

### TRAF6 activation of PI 3-kinase requires Ras function and Src kinase activity

To investigate the regulatory mechanisms for TRAF6 activation of PI 3-kinase, we focused on Ras, a member of the small G-protein family, and the protein tyrosine kinase catalytic activity of Src. The interest in Ras relates to its involvement in IL-1 signaling downstream of TRAF6 (Caunt et al., 2001) and the IRAK-dependent association of TRAF6, IRAK and TAK that occurs in response to IL-1 (McDermott and O'Neill, 2002; Takaesu et al., 2001). Following co-transfection of either TRAF6 or Src expression vectors with PH-Akt-GFP into HEK293 cells, a specific Ras farnesyl transferase inhibitor (FTI) at a concentration of 500 nM was added (Sepp-Lorenzino et al., 1995). Fig. 7 shows that FTI partially diminishes the activation of PI 3-kinase triggered either by TRAF6 or by Src with a resultant decrease in labeled filopodia (Fig. 7a,b,d,e). By contrast, when the co-transfected cells were treated with 500 nM of the PP2 Src kinase inhibitor, PI 3-kinase activity was severely reduced for both TRAF6 and Src (Fig. 7c,f). Treating TRAF6 and PH-Akt-GFP co-transfected cells with FTI plus PP2 completely inhibited PI 3-kinase activation, and filopodia are not observed (Fig. 7g). Furthermore, a kinase-deficient Src expression vector, Src (K298D), was incapable of activating PI 3-kinase, and when co-transfected with TRAF6, attenuated the TRAF6 activation of PI 3-kinase (Fig. 7h,i). These data suggest that the catalytic function of Src is crucial for the majority of PI 3-kinase activation, but a second Ras-dependent pathway appears to amplify the effect.

### TRAF6 activity correlates with non-sequestosomal subcellular localization

Previous reports using antibody staining of endogenous and GFP-tagged, ectopically expressed, TRAF proteins have consistently revealed intense punctate sequestosomes within the cytoplasm (Force et al., 2000; Sanz et al., 2000; Seibenhener et al., 2004; Zapata et al., 2001). In the present study, we have observed a similar phenomenon (Fig. 6D, Fig. 8A). It has been suggested that sequestosomes, which have been associated with ubiquitin proteasome degradation and TRAF6 binding to the PKCζ-interacting protein p62 (Seibenhener et al., 2004), are required for TRAF6 signaling (Wooten et al., 2005). This might be reasonable, since TRAF6 serves as an auto-ubiquitin ligase in a manner that appears to require the N-terminal RZF region for lysine 63 (K63)-linked polyubiquitylation and appears to be important for at least one NF-κB activation pathway (Kanayama et al., 2004). By contrast, our functional studies (Fig. 5) argue for the importance of the polyPro sequence in the C-terminal MATH domain for both NF-κB and PI 3-kinase activities. In addition, the BiFC studies argue for polyPro-dependent interaction between TRAF6 and Src in the non-sequestosomal cytoplasm (Fig. 6).

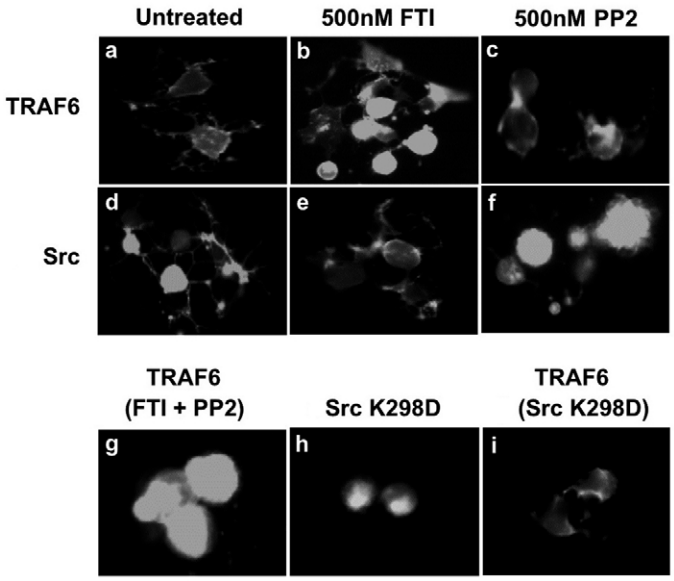
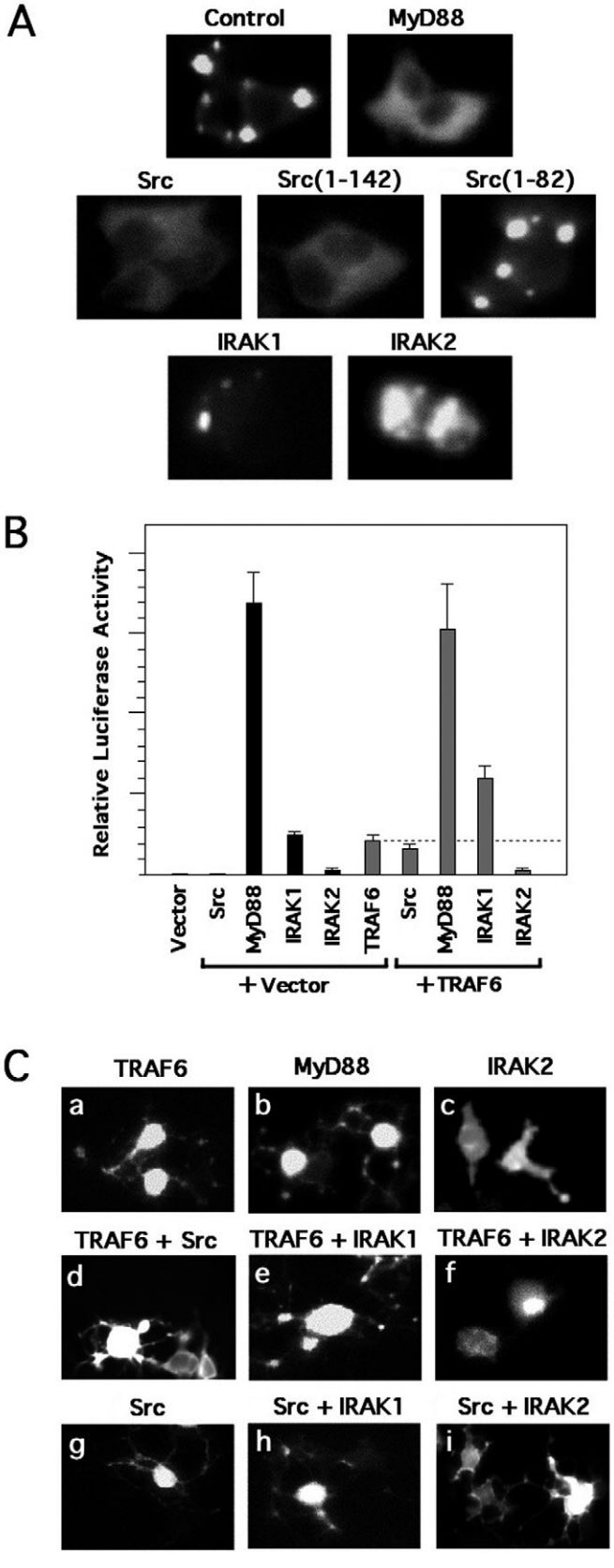



**Fig. 7.** Src and Ras mediate TRAF6 activation of PI 3-kinase. A PH-Akt-GFP expression vector reporter was co-transfected along with indicated plasmids into HEK293 cells. Src- and Ras-specific inhibitors were each added, as indicated above each panel, to a final concentration of 500 nM into individual wells at 6 hours. Images were taken 24 hours after transfection.

Therefore, we set out to determine whether the subcellular localization of transfected dominant-positive-acting TRAF6 can be affected by co-expression with other proteins reported to be involved in TRAF6 function (Boch et al., 2003; Medzhitov et al., 1998). The TRAF6 localization to the sequestosome normally observed for the transfected TRAF6-YFP expression vector (Fig. 8A, Control) was eliminated, resulting in TRAF6 relocalization to the cytoplasm by co-expression of vectors encoding either MyD88 or Src. Co-expression of either of these vectors with TRAF6 did not significantly reduce NF-κB activity, although MyD88 appeared to enhance the response (Fig. 8B). This contrasts with co-expressed IRAK2, which greatly enhanced TRAF6 sequestosome-like inclusions (Fig. 8A, IRAK2) and reduced both measured activities (Fig. 8B and Fig.8C, f). The truncated Src (1-142) construct containing the SH3 domain, like full-length Src, also effectively inhibited sequestosome formation and relocalized TRAF6 to the cytoplasm, whereas SH3-deficient Src (1-82) had no effect (Fig. 8A). This is consistent with the BiFC results shown in Fig. 6, which argue for the involvement of the SH3 domain in the interaction with TRAF6. Co-expression of a vector encoding IRAK1 did not significantly inhibit normal TRAF6 localization (Fig. 8A) or activities (Figs 8B and Fig. 8C, e). Individual expression of

either TRAF6 or MyD88 activated both NF-κB (Fig. 8B) and PI 3-kinase (Fig. 8C, a and b) activities, whereas Src expression in the absence of TRAF6 activated PI 3-kinase (8C, g), but not NF-κB (Fig. 8B). Distinctly, IRAK2 was only a very weak activator of these activities, and acted as a potent inhibitor of TRAF6-dependent activities (Fig. 8B and Fig. 8C, c and f). By contrast, IRAK2 did not inhibit Src-dependent activation of PI 3-kinase (Fig. 8C, i). Collectively, these data do not reveal a correlation between sequestosome formation and activity. Furthermore, the dominant-negative effect of IRAK2 on TRAF6-dependent activities and its ability to generate abnormally large sequestosome-like inclusions suggests that increased sequestosome formation might correlate with activity reduction. Therefore, it appears as though NF-κB and Src activities, as well as the TRAF6-Src interaction, do not necessarily correlate with sequestosomal localization of TRAF6. Furthermore, the inability of IRAK2 to inhibit PI 3-kinase activity induced by Src clearly places Src downstream of TRAF6.




## Discussion

The generation of cellular morphological changes such as filopodia formation by TRAF6 expression is important for several reasons. Our observation that TRAF6 can induce these changes is the first demonstration supporting the hypothesized role for this molecule in transducing the IL-1 signal for actin remodeling and crosslinking. Furthermore, the dependence upon PI 3-kinase activity, as revealed by LY-294002 inhibition, bolsters the argument for activation of actin branch formation through a WASP-like pathway involving Arp2/3 complex formation. This is consistent with our observation of PH-Akt-GFP recruitment at acute angular filopodia branch points, a hallmark of this process (Revenu et al., 2004). The involvement of Src kinase activity, as demonstrated by PP2 inhibition, and the requirement for an intact catalytic domain, argues in favor of a role for Src in generating tyrosine phosphorylation products essential for this process. As we and others have reported, ligand engagement results in the rapid tyrosine phosphorylation and recruitment of PI 3-kinase to analogous sites within the TIR domains of IL-1 and TLR receptors (Marmiroli et al., 1998; Reddy et al., 1997; Sarkar et al., 2004; Sizemore et al., 1999). Furthermore, we demonstrated previously that modification of a single tyrosine within the IL-$1R_I$ receptor TIR domain, responsible for direct recruitment of the p85 regulatory subunit of PI 3-kinase and indirect recruitment of the p110 catalytic subunit, resulted in a significant decrease in activity (Marmiroli et al., 1998).

**Fig. 8.** TRAF6 is relocalized when co-expressed with either an upstream or downstream activator. (A) Subcellular localization of TRAF6-YFP co-transfected with various expression vectors encoding non-labeled activator proteins. 70 ng total DNA (20 ng of TRAF6-YFP and 50 ng of expression vector, as indicated) were co-transfected into HEK293 cells. 'Control' represents cells co-transfected with 20 ng TRAF6-YFP and 50 ng of empty expression vector. Src, MyD88 and IRAK2 are wild-type expression vectors. Src(1-142) and Src(1-82) expression vectors contain the regions indicated in Fig. 4. (B) NF-κB activity resulting from co-transfection in HEK293 cells with expression vectors, as indicated, and the luciferase reporter described in Fig. 5. The amount of transfected vectors is the same as in Fig. 8A. A total of 70 ng of DNA was used for each transfection, including the empty 'Vector' control. (C) PH-Akt-GFP expression vector reporter was co-transfected together with cDNA vectors encoding proteins, as indicated above each panel, into HEK293 cells.

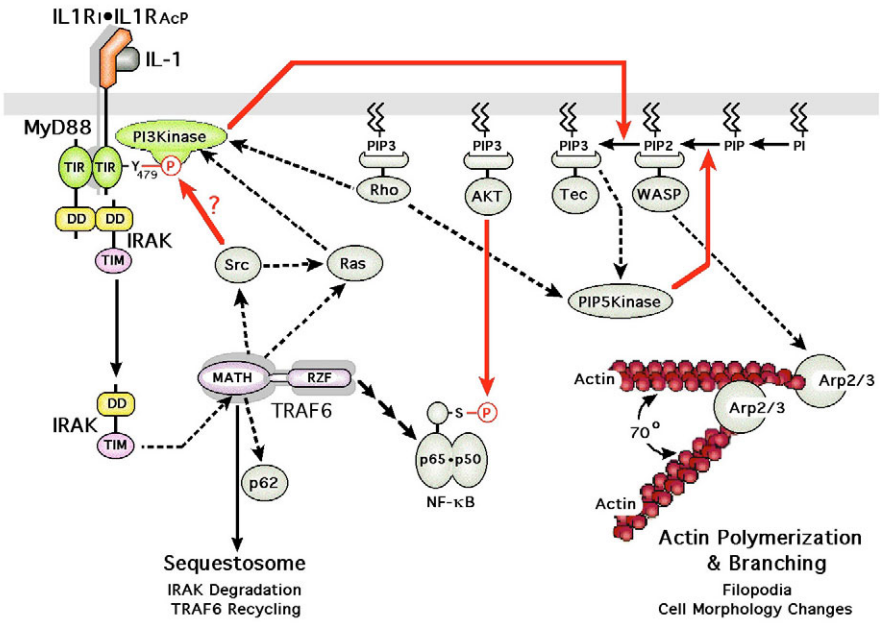



**Fig. 9.** Schematic representation of TRAF-dependent PI 3-kinase pathways. The data presented in this paper are consistent with a pathway involving both Ras and Src as key molecules in generating TRAF6 and PI 3-kinase-dependent activation of actin-mediated changes in cell morphology. The schematic incorporates relevant pathways derived from other reports as described in the text. Solid black arrows connect sequential pathway elements, red arrows designate enzymatic action, and broken arrows indicate protein-protein interactions resulting in a directional activation, as indicated by arrowheads. The activation of NF-κB by TRAF6 is indicated by tandem arrows, representative of the multistep process involving IκB kinase-activated phosphorylation and degradation of IκB resulting in subsequent release of active p50•p65. Abbreviations: $IL1R_I•IL1R_{AcP}$, IL-1 ligand-binding transmembrane receptor complex comprising the type I and accessory receptors; TIR, Toll-IL-1/18 homology domain; DD, death domain; TIM, TRAF-interaction motif; WASP, Wiskott-Aldrich syndrome protein; PI, phosphatidylinositol; PIP, phosphatidylinositol 4-monophosphate; PIP2, phosphatidylinositol (4,5)-bisphosphate [PtdIns(4,5)$P_2$]; PIP3, phosphatidylinositol (3,4,5)-trisphosphate [PtdIns(3,4,5)$P_3$]; PIP5Kinase, phosphatidylinositol-4-phosphate 5-kinase.

Consequently, TIR receptors could serve as targets for Src kinase function (Fig. 9), resulting in membrane-proximal recruitment of PI 3-kinase and the generation of PtdIns(3,4,5)$P_3$ and PIP2 (Kane and Weiss, 2003).

Since the PH domain of Akt binds PtdIns(3,4,5)$P_3$ with very high affinity ($K_a$~$10^8$ M) and both diphosphate forms of PtdIns(3,4)$P_2$ and PtdIns(4,5)$P_2$ at a lower, but significant, affinity (James et al., 1996), it has been demonstrated to be a capable reporter of PI 3-kinase product formation (Kane et al., 2004). We have taken advantage of this approach to assay PI 3-kinase activity in living cells. It should be noted that even though PtdIns(4,5)$P_2$ is a substrate, rather than a product, of PI 3-kinase, its levels are not very high until PI 3-kinase is activated, resulting in membrane recruitment and activation of either Tec kinases or Rho-family GTPases by PtdIns(3,4,5)$P_3$ (Fig. 9) (Weernink et al., 2004). The PH domain of the Tec-family kinases Tec and Btk mediate a direct interaction with phosphatidylinositol-4-phosphate 5-kinase (Saito et al., 2003) upstream of PI 3-kinase. This increases synthesis of the PtdIns(4,5)$P_2$ diphosphate form, resulting in greater synthesis of downstream PtdIns(3,4,5)$P_3$ (Saito et al., 2003). Furthermore, PtdIns(3,4,5)$P_3$ activates Rho-family GTPases that, like the Tec kinases, activate phosphatidylinositol-4-phosphate 5-kinase (Saci and Carpenter, 2005; Yang et al., 2004). Consequently, PH-Akt-GFP recruitment may not only represent the presence of direct PI 3-kinase synthesis products, but also of an indirect product, such as PtdIns(4,5)$P_2$, which has been shown to recruit and activate WASP-like proteins (Fig. 9) through interaction with the WASP polybasic auto-inhibitory domain (Millard et al., 2004; Papayannopoulos et al., 2005). Our observation of a TRAF6-dependent activation of Ras (Fig. 7) is consistent with reports that Ras functions downstream of TRAF6 in IL-1 signaling (Caunt et al., 2001), and that IRAK-dependent association of TRAF6, IRAK and TAK occurs in response to IL-1 (McDermott and O'Neill, 2002; Takaesu et al., 2001).

Ligands for TIR-family receptors, such as IL-1 and lipopolysaccharide, as well as ligands for some TNFRs, such as RANK ligand, nerve growth factor and CD40 ligand, initiate cellular pleiotropic responses that are mediated to a major degree by the activation of TRAF6. Although TRAF6 appears to be cytoplasmic prior to activation, it has been reported to be translocated to the cell membrane following ligand engagement by those TNFRs that directly recruit various TRAFs through intracellular interaction motifs (Ha et al., 2003; Hostager et al., 2000; Vidalain et al., 2000; Wong et al., 1999; Wooten et al., 2001). However, for some TNFRs, as well as TIR, activated TRAF6 appears to be associated with large, non-membrane-bound molecular aggregates called sequestosomes (Puls et al., 1997; Sanz et al., 2000; Seibenhener et al., 2004). The sequestosome, which specifically localizes polyubiquitin, IRAK, TRAF6 and PKCζ, also contains 20S proteasomes (Donaldson et al., 2003; Sanz et al., 2000; Seibenhener et al., 2004) and might represent a proteasome, aggregating, but inefficiently degrading, ubiquitylated proteins (Stumptner et al., 2002). TRAF6 functions as an autocatalytic Ring-finger-dependent K63 ubiquitin ligase, upon which RZF-dependent NF-κB and MAPK activation appears to depend (Kanayama et al., 2004; Wooff et al., 2004). In addition, in vitro cell-free phosphorylation of IκBα has been reported to be associated with high-molecular-weight aggregates involving multimers of TRAF6 and the TRAF6-IRAK linking-protein TIFA (Ea et al., 2004; Takatsuna et al., 2003), and IL-1 treatment of cells ectopically expressing p62 has revealed relocalization of TRAF6 into sequestosomes. Taken together with reports demonstrating that p62 is a crucial adaptor for NF-κB activation through atypical PKC such as PKCζ (Duran et al., 2004; Leitges et al., 2001), it might appear as though TRAF6 signal transduction is mediated by sequestosomal association. However, this topic is somewhat controversial in that conflicting reports argue either for an absolutely particulate

nature for p62 (Sanchez et al., 1998) or, like TRAF6, p62 residence both in and out of the sequestosome (Puls et al., 1997). Adding to the controversy, these same reports argue that the crucial PKCζ component with which TRAF6 and p62 simultaneously associate is exclusively localized to one or the other subcellular location. Therefore, it is likely that p62, IRAK, TRAF6 and PKCζ exist in both locations. Consequently, it is unclear whether the sequestosome is the exclusive site of TRAF6 signaling or is part of a second pathway. However, our observations collectively argue that signaling probably occurs within the bulk cytoplasm and not within the sequestosome. These observations include: direct interaction between Src and TRAF6 outside of the sequestosome; inhibition of TRAF6-dependent sequestosome formation by dominant-positive levels of either Src or MyD88; and greatly increased prominence of sequestosomes in the presence of inhibitory levels of IRAK2 that correlates with both NF-κB and PI 3-kinase activities.

It is possible that the role of the sequestosome might be to accumulate activated p62 and IRAK-associated TRAF6 for recycling through a proteosomal pathway. This hypothesis might appear to be inconsistent with reports that K63 polyubiquitylation of TRAF6 is required for TAB-TAK-dependent NF-κB activation (Kanayama et al., 2004) and that TRAF6 is recycled by deubiquitylation, rather than by degradation (Jensen and Whitehead, 2003; Trompouki et al., 2003). However, it could also be argued that the sequestosome serves as a site of sequestration for inactive or post-activated TRAF6. This would be analogous to the non-membrane-bound sequestosome-like structures that recruit a complex between the p85 regulatory subunit of PI 3-kinase and phosphorylated IRIS-1, following cell treatment with insulin-like growth factor 1 (Luo et al., 2005). This could explain why the size of both p62 and TRAF6 sequestosomes increases following either IL-1 activation or the treatment of cells with an inhibitor of proteasome function (Seibenhener et al., 2004), and why p62 and TRAF6 serve as a mediators of K63 polyubiquitylated Tau protein degradation by proteosomes in neurodegenerative diseases (Babu et al., 2005). Consequently, activation by either IL-1 stimulation or TRAF6 overexpression could first result in cytoplasmic activation of Src and/or PI 3-kinase and NF-κB pathways, followed by recruitment of K63 polyubiquitylated TRAF6 to the sequestosome, where a proteasome-function-dependent process might be required for TRAF6 to be recycled. Such a process could involve the recycling of TRAF6 by deubiquitylation in concert with proteosomal degradation of associated factors such as p62 and IRAK1. Supporting this model, immediately following either IL-1 or TLR signal transduction, a TIR cross-ligand tolerance signaling desensitization occurs as a result of rapid IRAK1 degradation (Li et al., 2000; Yamin and Miller, 1997). This model would not ignore the importance of K63 polyubiquitylation in signal transduction, since it does appear to be required for such processes as the interaction of TAB-TAK with TRAF, resulting in downstream TAK-dependent signaling (Kanayama et al., 2004). This model is also consistent with a lack of cross-desensitization between TIR and TNFR (Medvedev et al., 2000), since TRAF6 is recycled and IRAK is not associated with TNFR signaling.

The complete elimination of TRAF6 sequestosomes by co-expression of full-length MyD88 could be a result of the presence of the TIR homology domain, which forms homotypic interactions with the TIR domains of IL-1Rs. Such an interaction might alter the subcellular localization of IRAK1 from the sequestosome, where it has been reported to co-localize with TRAF6 and p62 (Sanz et al., 2000), to the vicinity of the cell membrane. By contrast, the super-accumulation of sequestosomes and inhibition of TRAF6 dominant-positive activity by IRAK2 is striking. Since IRAK2 can serve as a weak dominant positive in the absence of ligand stimulation, but inhibits activation by ectopically expressed TRAF6 (Fig. 8B), its steady-state level within the cell might be crucial. Interestingly, it has been reported that IRAK2 is not susceptible to stimulation-dependent degradation (Hu et al., 2002b), suggesting that non-degradable IRAK2 might irreversibly recruit TRAF6 to the sequestosome. Finally, the Src-dependent inhibition of sequestosomes, like the interaction between TRAF6 and Src, appears to be dependent upon the presence of the Src SH3 domain (Fig. 6, Fig. 8A). This further supports a role for polyPro interactions in TRAF6 function.

The presence of Src within the cytoplasm is consistent with reports that this myristoylated protein is not exclusively localized to the cell membrane in many cells. The Src-family kinases are all either mono- or di-acylated at their N-termini, and only the di-acylated forms appear to be constitutively localized to cell membranes. Mono-acylation does not ensure cell membrane targeting. For example, both human and mouse Hck, a Src-family kinase, exist as two alternatively translated forms with distinct acylation patterns. Mono-acylated myristoyl-Hck resides primarily in the cytoplasm, whereas the di-acylated myristoyl-palmitoyl form is exclusively associated with the cell membrane (Carreno et al., 2000; Lock et al., 1991). The subcellular location of Src has specifically been shown to be predominantly cytoplasmic, relocalizing to the cell membrane following activation by platelet-derived growth factor (PDGF) (Ochoa et al., 2000; Sandilands et al., 2004). Consequently, Src is probably available within the cell for interaction with TRAF6. Specifically, it has been reported that cytoplasmic Src in osteoclasts rapidly co-localizes with TRAF6 following IL-1 treatment (Nakamura et al., 2002). A somewhat similar situation occurs for Ras, a protein that cycles between mono- and multi-acylated forms, resulting in cytoplasmic as well as membrane localization (Rocks et al., 2005). Although the interaction between TRAF6 and Src might occur in the cytoplasm, the activation of PI 3-kinase would still be expected to depend upon downstream events within the membrane and maintenance of the membrane microenvironment, as supported by the observation of inhibition by MbCD (Fig. 2B).

It has been reported that PI 3-kinase and Akt are involved in IKK-mediated activation of NF-κB through the T-cell receptor or following oxidative stress (Fan et al., 2003; Kang et al., 2003; Lee et al., 2005; Ozes et al., 1999; Storz et al., 2004), as well as from events such as the direct phosphorylation of the NF-κB p65 subunit (Auron, 1998; Madrid et al., 2001; Marmiroli et al., 1998), which manifest activity differences only following IKK-dependent activation. We previously reported that IL-1 activation of NF-κB activity is reduced approximately 50% in human Saos2 cells (Marmiroli et al., 1998). We now show that NF-κB activity resulting from ectopic TRAF6 expression is also reduced both by catalytic inhibition and by mutation of the Src-interaction motif on TRAF6 (Fig. 5B). However, Src

expression, with presumable PI 3-kinase function, did not activate NF-κB (Fig. 8B), whereas dominant-positive PI 3-kinase p110 did (Marmiroli et al., 1998). This suggests that Src is necessary, but insufficient, for maximum NF-κB activation. It could be that the level of PI 3-kinase activity is crucial and that Ras or some other second signal provided by TRAF6 is required. Fig. 9 schematically summarizes and attempts to integrate the apparently diverse, but related, observations resulting from our studies and those of others referenced above. This model places TRAF6 within the cytoplasm where, in the absence of membrane recruitment, it might be able to activate downstream targets that can, in turn, activate PI 3-kinase. We also propose that the sequestosome might be involved in TRAF recycling, rather than activation. Such a model positions IRAK in the role of both activating TRAF6 for signaling within the cytoplasm and subsequently targeting it to the sequestosome for the previously reported degradation of IRAK1 and TRAF recycling. In this way, TRAF6 is made available for TNFR signaling, whereas IRAK1-dependent TIR signaling is desensitized.

## Materials and Methods

### Chemicals

Phalloidin-TRITC (tetramethylrhodamine isothiocyanate), methyl-β cyclodextrin (MbCD) and cytochalasin D were purchased from Sigma Aldrich. The LY294002, PP2 and farnysyl transferase inhibitor 1 (FTI-1/B581) were from Calbiochem. Interleukin 1β (IL-1) was purchased from Upstate Biotech.

### Plasmid constructs

TRAF6 was cloned in PCDNA3.1 by PCR as described previously (Yoshida et al., 2004). TRAF6-P465A, double-substituted TRAF6-P2A (P465A, P468A), and triple-substituted TRAF6-P3A (P462A, P465A, P468A) were generated using a Stratagene QuickChange XL site-directed mutagenesis kit. Human Src and kinase-deficient SrcK298D plasmids were kindly provided by T. E. Smithgall (University of Pittsburgh, Pittsburgh, PA). The constitutively active catalytic subunit of PI 3-kinase (P110*) and plekstrin-homology domain of Akt conjugated with GFP (PH-Akt-GFP) plasmids were kind gifts from L. P. Kane (University of Pittsburgh, Pittsburgh, PA). Expression vectors encoding YFP fusions with TRAF6 and Src derivatives were generated by recombinant PCR techniques and inserted into a pFLAG CMV5a vector (Sigma).

### Cell lines

HEK293 (CRL-1573), HEK293T (CRL-11268), Hep3B (HB-8064) and RAW264.7 (TIB-71) cells were from American Type Culture Collection. The HEK293R was derived from HEK293 by stable integration of a cDNA expressing the IL-1 type I receptor (IL-1$R_I$) chain (Yoshida et al., 2004). HEK293 and Hep3B cells were maintained in EMEM medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mg/ml L-glutamine. RAW264.7 cells were maintained in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum and 2 mg/ml L-glutamine.

### Cell transfection

Cells were seeded into 96-well plates (flat bottoms) 24 hours before transfection. Indicated plasmids were mixed with FuGene 6 (Roche) according to the manufacturer's instructions. Transfected cells were treated by IL-1 or inhibitors after 24 hours of transfection.

### Microscopy

Cells were examined and images acquired using a Zeiss Axiovert 200M microscope equipped with a 100 W halogen light source, Zeiss Axiocam MRm cooled camera, and Zeiss Axiovision software. Epifluorescence experiments used Chroma Technology filter sets 41003 (for TRITC) and 41017 (for GFP and YFP).

### F-actin staining

Cells were fixed with 2% glutaraldehyde in PBS at room temperature for 15 minutes and permeabilized in 1% Triton X-100 at room temperature for 5 minutes, then stained with 1 μg/ml phalloidin-TRITC at room temperature for 45 minutes according to the manufacturer's protocol.

### Luciferase activity

TRAF6 cDNA expression constructs, as indicated in the figure legends, were co-transfected with 60 ng of a classic NF-κB site reporter, MHCκB-pGL2, into HEK293 cells for 24 hours, after which cells were then lyzed for luciferase activity and analysis as described previously (Yoshida et al., 2004). Each sample was independently transfected a minimum of three times and then analyzed in triplicate. Data presented was derived from a single representative transfection with triplicate values presented as bars representing s.e.m. Activities of TRAF6 constructs were interpreted as fold induction normalized by comparison with the empty control expression vector.

### Bimolecular fluorescence complementation (BiFC)

The cDNA corresponding to the YN amino fragment (aa 1-154) of YFP was inserted into pFlag-CMV-5a (Sigma-Aldrich) at *Eco*RV and *Bam*HI sites by PCR. The pHA-YC plasmid, which expresses the carboxyl fragment of YFP (aa 155-238) was constructed as previously described (Hu et al., 2002a). TRAF6-YC, TRAF6-P465A-YC and TRAF6-P3A-YC were constructed by PCR and inserted into the *Sal*I and *Kpn*I sites of pHA-YC. A linker of (Gly-Gly-Gly-Gly-Ser)×4 was inserted between the C-terminus of TRAF6 and BiFC cDNA sequences using PCR. Src-YN, Src(1-142)-YN and Src(1-82)-YN were constructed in the pFLAG CMV5a-YN vector at *Eco*RI and *Bgl*II sites using the same strategy as above. Protein-protein interaction in living cells was tested by co-transfecting TRAF6-YC and Src-YN or other combinations into HEK293T cells. Cells were examined and images acquired after 24 hours of transfection, as described above.

We thank Y. Koyama and A. Arman for confirming investigations. L. P. Kane and T. E. Smithgall are acknowledged for critical reading of the manuscript and for providing several vectors used in this study. This work has been supported in part by grants from the Pennsylvania Tobacco Settlement Fund to P.E.A. and from the National Institutes of Health to P.E.A. (CA68544 and AI44122) and J.A.B. (DE14197).

## References

**Akira, S. and Takeda, K.** (2004). Toll-like receptor signalling. *Nat. Rev. Immunol*. **4**, 499-511.

**Auron, P. E.** (1998). The interleukin 1 receptor: ligand interactions and signal transduction. *Cytokine Growth Factor Rev.* **9**, 221-237.

**Babu, J. R., Geetha, T. and Wooten, M. W.** (2005). Sequestosome 1/p62 shuttles polyubiquitinated tau for proteasomal degradation. *J. Neurochem.* **94**, 192-203.

**Baud, V., Liu, Z. G., Bennett, B., Suzuki, N., Xia, Y. and Karin, M.** (1999). Signaling by proinflammatory cytokines: oligomerization of TRAF2 and TRAF6 is sufficient for JNK and IKK activation and target gene induction via an amino-terminal effector domain. *Genes Dev.* **13**, 1297-1308.

**Boch, J. A., Yoshida, Y., Koyama, Y., Wara-Aswapati, N., Peng, H., Unlu, S. and Auron, P. E.** (2003). Characterization of a cascade of protein interactions initiated at the IL-1 receptor. *Biochem. Biophys. Res. Commun.* **303**, 525-531.

**Brown, K. D., Hostager, B. S. and Bishop, G. A.** (2001). Differential signaling and tumor necrosis factor receptor-associated factor (TRAF) degradation mediated by CD40 and the Epstein-Barr virus oncoprotein latent membrane protein 1 (LMP1). *J. Exp. Med.* **193**, 943-954.

**Cao, Z., Xiong, J., Takeuchi, M., Kurama, T. and Goeddel, D. V.** (1996). TRAF6 is a signal transducer for interleukin-1. *Nature* **383**, 443-446.

**Carreno, S., Gouze, M. E., Schaak, S., Emorine, L. J. and Maridonneau-Parini, I.** (2000). Lack of palmitoylation redirects p59Hck from the plasma membrane to p61Hck-positive lysosomes. *J. Biol. Chem.* **275**, 36223-36229.

**Caunt, C. J., Kiss-Toth, E., Carlotti, F., Chapman, R. and Qwarnstrom, E. E.** (2001). Ras controls tumor necrosis factor receptor-associated factor (TRAF)6-dependent induction of nuclear factor-kappa b. Selective regulation through receptor signaling components. *J. Biol. Chem.* **276**, 6280-6288.

**Dempsey, P. W., Doyle, S. E., He, J. Q. and Cheng, G.** (2003). The signaling adaptors and pathways activated by TNF superfamily. *Cytokine Growth Factor Rev.* **14**, 193-209.

**Donaldson, K. M., Li, W., Ching, K. A., Batalov, S., Tsai, C. C. and Joazeiro, C. A.** (2003). Ubiquitin-mediated sequestration of normal cellular proteins into polyglutamine aggregates. *Proc. Natl. Acad. Sci. USA* **100**, 8892-8897.

**Dunne, A. and O'Neill, L. A.** (2003). The interleukin-1 receptor/Toll-like receptor superfamily: signal transduction during inflammation and host defense. *Sci STKE*

**Duran, A., Serrano, M., Leitges, M., Flores, J. M., Picard, S., Brown, J. P., Moscat, J. and Diaz-Meco, M. T.** (2004). The atypical PKC-interacting protein p62 is an important mediator of RANK-activated osteoclastogenesis. *Dev. Cell* **6**, 303-309.

**Ea, C. K., Sun, L., Inoue, J. and Chen, Z. J.** (2004). TIFA activates IkappaB kinase (IKK) by promoting oligomerization and ubiquitination of TRAF6. *Proc. Natl. Acad. Sci. USA* **101**, 15318-15323.

**Fan, C., Li, Q., Ross, D. and Engelhardt, J. F.** (2003). Tyrosine phosphorylation of I kappa B alpha activates NF kappa B through a redox-regulated and c-Src-dependent mechanism following hypoxia/reoxygenation. *J. Biol. Chem.* **278**, 2072-2080.

**Force, W. R., Glass, A. A., Benedict, C. A., Cheung, T. C., Lama, J. and Ware, C. F.** (2000). Discrete signaling regions in the lymphotoxin-beta receptor for tumor necrosis factor receptor-associated factor binding, subcellular localization, and activation of cell death and NF-kappaB pathways. *J. Biol. Chem.* **275**, 11121-11129.

**Gravallese, E. M., Galson, D. L., Goldring, S. R. and Auron, P. E.** (2001). The role of TNF-receptor family members and other TRAF-dependent receptors in bone resorption. *Arthritis Res*. **3**, 6-12.

**Ha, H., Kwak, H. B., Lee, S. K., Na, D. S., Rudd, C. E., Lee, Z. H. and Kim, H. H.** (2003). Membrane rafts play a crucial role in receptor activator of nuclear factor kappaB signaling and osteoclast function. *J. Biol. Chem*. **278**, 18573-18580.

**Hostager, B. S., Catlett, I. M. and Bishop, G. A.** (2000). Recruitment of CD40 and tumor necrosis factor receptor-associated factors 2 and 3 to membrane microdomains during CD40 signaling. *J. Biol. Chem*. **275**, 15392-15398.

**Hu, C. D., Chinenov, Y. and Kerppola, T. K.** (2002a). Visualization of interactions among bZIP and Rel family proteins in living cells using bimolecular fluorescence complementation. *Mol. Cell* **9**, 789-798.

**Hu, J., Jacinto, R., McCall, C. and Li, L.** (2002b). Regulation of IL-1 receptor-associated kinases by lipopolysaccharide. *J. Immunol*. **168**, 3910-3914.

**James, S. R., Downes, C. P., Gigg, R., Grove, S. J., Holmes, A. B. and Alessi, D. R.** (1996). Specific binding of the Akt-1 protein kinase to phosphatidylinositol 3,4,5-trisphosphate without subsequent activation. *Biochem. J*. **315**, 709-713.

**Janmey, P. A. and Lindberg, U.** (2004). Cytoskeletal regulation: rich in lipids. *Nat. Rev. Mol. Cell Biol*. **5**, 658-666.

**Jensen, L. E. and Whitehead, A. S.** (2003). Ubiquitin activated tumor necrosis factor receptor associated factor-6 (TRAF6) is recycled via deubiquitination. *FEBS Lett*. **553**, 190-194.

**Jeschke, M., Brandi, M. L. and Susa, M.** (1998). Expression of Src family kinases and their putative substrates in the human preosteoclastic cell line FLG 29.1. *J. Bone Miner. Res*. **13**, 1880-1889.

**Kanayama, A., Seth, R. B., Sun, L., Ea, C. K., Hong, M., Shaito, A., Chiu, Y. H., Deng, L. and Chen, Z. J.** (2004). TAB2 and TAB3 activate the NF-kappaB pathway through binding to polyubiquitin chains. *Mol. Cell* **15**, 535-548.

**Kane, L. P. and Weiss, A.** (2003). The PI-3 kinase/Akt pathway and T cell activation: pleiotropic pathways downstream of PIP3. *Immunol. Rev.* **192**, 7-20.

**Kane, L. P., Mollenauer, M. N. and Weiss, A.** (2004). A proline-rich motif in the C terminus of Akt contributes to its localization in the immunological synapse. *J. Immunol*. **172**, 5441-5449.

**Kang, J. L., Lee, H. S., Pack, I. S., Hur, K. C. and Castranova, V.** (2003). Phosphoinositide 3-kinase activity leads to silica-induced NF-kappaB activation through interacting with tyrosine-phosphorylated I(kappa)B-alpha and contributing to tyrosine phosphorylation of p65 NF-kappaB. *Mol. Cell. Biochem*. **248**, 17-24.

**Kobayashi, N., Kadono, Y., Naito, A., Matsumoto, K., Yamamoto, T., Tanaka, S. and Inoue, J.** (2001). Segregation of TRAF6-mediated signaling pathways clarifies its role in osteoclastogenesis. *EMBO J*. **20**, 1271-1280.

**Kopp, E., Medzhitov, R., Carothers, J., Xiao, C., Douglas, I., Janeway, C. A. and Ghosh, S.** (1999). ECSIT is an evolutionarily conserved intermediate in the Toll/IL-1 signal transduction pathway. *Genes Dev*. **13**, 2059-2071.

**Layfield, R. and Hocking, L. J.** (2004). SQSTM1 and Paget's disease of bone. *Calcif. Tissue Int.* **75**, 347-357.

**Lee, H. K., Dunzendorfer, S. and Tobias, P. S.** (2004a). Cytoplasmic domain-mediated dimerizations of toll-like receptor 4 observed by beta -lactamase enzyme fragment complementation. *J. Biol. Chem*. **279**, 10564-10574.

**Lee, H. T., Lee, J. G., Na, M. and Kay, E. P.** (2004b). FGF-2 induced by interleukin-1 beta through the action of phosphatidylinositol 3-kinase mediates endothelial mesenchymal transformation in corneal endothelial cells. *J. Biol. Chem*. **279**, 32325-32332.

**Lee, K. Y., D'Acquisto, F., Hayden, M. S., Shim, J. H. and Ghosh, S.** (2005). PDK1 nucleates T cell receptor-induced signaling complex for NF-kappaB activation. *Science* **308**, 114-118.

**Leitges, M., Sanz, L., Martin, P., Duran, A., Braun, U., Garcia, J. F., Camacho, F., Diaz-Meco, M. T., Rennert, P. D. and Moscat, J.** (2001). Targeted disruption of the zetaPKC gene results in the impairment of the NF-kappaB pathway. *Mol. Cell* **8**, 771-780.

**Li, L., Cousart, S., Hu, J. and McCall, C. E.** (2000). Characterization of interleukin-1 receptor-associated kinase in normal and endotoxin-tolerant cells. *J. Biol. Chem*. **275**, 23340-23345.

**Lock, P., Ralph, S., Stanley, E., Boulet, I., Ramsay, R. and Dunn, A. R.** (1991). Two isoforms of murine hck, generated by utilization of alternative translational initiation codons, exhibit different patterns of subcellular localization. *Mol. Cell. Biol*. **11**, 4363-4370.

**Locksley, R. M., Killeen, N. and Lenardo, M. J.** (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. *Cell* **104**, 487-501.

**Lowell, C. A., Niwa, M., Soriano, P. and Varmus, H. E.** (1996). Deficiency of the Hck and Src tyrosine kinases results in extreme levels of extramedullary hematopoiesis. *Blood* **87**, 1780-1792.

**Luo, J., Field, S. J., Lee, J. Y., Engelman, J. A. and Cantley, L. C.** (2005). The p85 regulatory subunit of phosphoinositide 3-kinase down-regulates IRS-1 signaling via the formation of a sequestration complex. *J. Cell Biol.* **170**, 455-464.

**Madrid, L. V., Mayo, M. W., Reuther, J. Y. and Baldwin, A. S., Jr** (2001). Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. *J. Biol. Chem*. **276**, 18934-18940.

**Marmiroli, S., Bavelloni, A., Faenza, I., Sirri, A., Ognibene, A., Cenni, V., Tsukada, J., Koyama, Y., Ruzzene, M., Ferri, A. et al.** (1998). Phosphatidylinositol 3-kinase is recruited to a specific site in the activated IL-1 receptor I. *FEBS Lett.* **438**, 49-54.

**Matsuo, K., Galson, D. L., Zhao, C., Peng, L., Laplace, C., Wang, K. Z., Bachler, M. A., Amano, H., Aburatani, H., Ishikawa, H. et al.** (2004). Nuclear factor of activated T-cells (NFAT) rescues osteoclastogenesis in precursors lacking c-Fos. *J. Biol. Chem.* **279**, 26475-26480.

**McDermott, E. P. and O'Neill, L. A.** (2002). Ras participates in the activation of p38 MAPK by interleukin-1 by associating with IRAK, IRAK2, TRAF6, and TAK-1. *J. Biol. Chem*. **277**, 7808-7815.

**Medvedev, A. E., Kopydlowski, K. M. and Vogel, S. N.** (2000). Inhibition of lipopolysaccharide-induced signal transduction in endotoxin-tolerized mouse macrophages: dysregulation of cytokine, chemokine, and toll-like receptor 2 and 4 gene expression. *J. Immunol*. **164**, 5564-5574.

**Medzhitov, R., Preston-Hurlburt, P., Kopp, E., Stadlen, A., Chen, C., Ghosh, S. and Janeway, C. A., Jr** (1998). Myd88 is an adaptor protein in the htoll/IL-1 receptor family signaling pathways. *Mol. Cell* **2**, 253-258.

**Michnick, S. W.** (2001). Exploring protein interactions by interaction-induced folding of proteins from complementary peptide fragments. *Curr. Opin. Struct. Biol*. **11**, 472-477.

**Millard, T. H., Sharp, S. J. and Machesky, L. M.** (2004). Signalling to actin assembly via the WASP (Wiskott-Aldrich syndrome protein)-family proteins and the Arp2/3 complex. *Biochem. J*. **380**, 1-17.

**Moscat, J., Sanz, L., Sanchez, P. and Diaz-Meco, M. T.** (2001). Regulation and role of the atypical PKC isoforms in cell survival during tumor transformation. *Adv. Enzyme Regul*. **41**, 99-120.

**Nakamura, I., Kadono, Y., Takayanagi, H., Jimi, E., Miyazaki, T., Oda, H., Nakamura, K., Tanaka, S., Rodan, G. A. and Duong, L. T.** (2002). IL-1 regulates cytoskeletal organization in osteoclasts via TNF receptor-associated factor 6/c-Src complex. *J. Immunol*. **168**, 5103-5109.

**Ochoa, G. C., Slepnev, V. I., Neff, L., Ringstad, N., Takei, K., Daniell, L., Kim, W., Cao, H., McNiven, M., Baron, R. et al.** (2000). A functional link between dynamin and the actin cytoskeleton at podosomes. *J. Cell Biol*. **150**, 377-389.

**Ozes, O. N., Mayo, L. D., Gustin, J. A., Pfeffer, S. R., Pfeffer, L. M. and Donner, D. B.** (1999). NF-kappaB activation by tumour necrosis factor requires the Akt serine-threonine kinase. *Nature* **401**, 82-85.

**Papayannopoulos, V., Co, C., Prehoda, K. E., Snapper, S., Taunton, J. and Lim, W. A.** (2005). A polybasic motif allows N-WASP to act as a sensor of PIP(2) density. *Mol. Cell* **17**, 181-191.

**Park, Y. C., Burkitt, V., Villa, A. R., Tong, L. and Wu, H.** (1999). Structural basis for self-association and receptor recognition of human TRAF2. *Nature* **398**, 533-538.

**Puls, A., Schmidt, S., Grawe, F. and Stabel, S.** (1997). Interaction of protein kinase C zeta with ZIP, a novel protein kinase C-binding protein. *Proc. Natl. Acad. Sci. USA* **94**, 6191-6196.

**Puls, A., Eliopoulos, A. G., Nobes, C. D., Bridges, T., Young, L. S. and Hall, A.** (1999). Activation of the small GTPase Cdc42 by the inflammatory cytokines TNF(alpha) and IL-1, and by the Epstein-Barr virus transforming protein LMP1. *J. Cell Sci*. **112**, 2983-2992.

**Reddy, S. A. G., Huang, J. H. and Liao, W. S. L.** (1997). Phosphatidylinositol 3-kinase in interleukin 1 signaling. Physical interaction with the interleukin 1 receptor and requirement in NFκB and AP-1 activation. *J. Biol. Chem.* **272**, 29167-29173.

**Revenu, C., Athman, R., Robine, S. and Louvard, D.** (2004). The co-workers of actin filaments: from cell structures to signals. *Nat. Rev. Mol. Cell Biol*. **5**, 635-646.

**Rocks, O., Peyker, A., Kahms, M., Verveer, P. J., Koerner, C., Lumbierres, M., Kuhlmann, J., Waldmann, H., Wittinghofer, A. and Bastiaens, P. I.** (2005). An acylation cycle regulates localization and activity of palmitoylated Ras isoforms. *Science* **307**, 1746-1752.

**Saci, A. and Carpenter, C. L.** (2005). RhoA GTPase regulates B cell receptor signaling. *Mol. Cell* **17**, 205-214.

**Saito, K., Tolias, K. F., Saci, A., Koon, H. B., Humphries, L. A., Scharenberg, A., Rawlings, D. J., Kinet, J. P. and Carpenter, C. L.** (2003). BTK regulates PtdIns-4,5-P2 synthesis: importance for calcium signaling and PI3K activity. *Immunity* **19**, 669-678.

**Sanchez, P., De Carcer, G., Sandoval, I. V., Moscat, J. and Diaz-Meco, M. T.** (1998). Localization of atypical protein kinase C isoforms into lysosome-targeted endosomes through interaction with p62. *Mol. Cell. Biol*. **18**, 3069-3080.

**Sandilands, E., Cans, C., Fincham, V. J., Brunton, V. G., Mellor, H., Prendergast, G. C., Norman, J. C., Superti-Furga, G. and Frame, M. C.** (2004). RhoB and actin polymerization coordinate Src activation with endosome-mediated delivery to the membrane. *Dev. Cell* **7**, 855-869.

**Sanz, L., Diaz-Meco, M. T., Nakano, H. and Moscat, J.** (2000). The atypical PKC-interacting protein p62 channels NF-kappaB activation by the IL-1-TRAF6 pathway. *EMBO J*. **19**, 1576-1586.

**Sarkar, S., Peters, K. L., Elco, C. P., Sakamoto, S., Pal, S. and Sen, G. C.** (2004). Novel roles of TLR3 tyrosine phosphorylation and PI3 kinase in double-stranded. *Nat. Struct. Mol. Biol.* **11**, 1028-1030.

**Scheiffele, P., Roth, M. G. and Simons, K.** (1997). Interaction of influenza virus haemagglutinin with sphingolipid-cholesterol membrane domains via its transmembrane domain. *EMBO J*. **16**, 5501-5508.

**Seibenhener, M. L., Babu, J. R., Geetha, T., Wong, H. C., Krishna, N. R. and Wooten, M. W.** (2004). Sequestosome 1/p62 is a polyubiquitin chain binding protein involved in ubiquitin proteasome degradation. *Mol. Cell. Biol*. **24**, 8055-8068.

**Sepp-Lorenzino, L., Ma, Z., Rands, E., Kohl, N. E., Gibbs, J. B., Oliff, A. and Rosen, N.** (1995). A peptidomimetic inhibitor of farnesyl:protein transferase blocks the anchorage-dependent and –independent growth of human tumor cell lines. *Cancer Res.* **55**, 5302-5309.

**Sizemore, N., Leung, S. and Stark, G. R.** (1999). Activation of phosphatidylinositol 3-kinase in response to interleukin-1 leads to phosphorylation and activation of the NF-kappaB p65/RelA subunit. *Mol. Cell. Biol*. **19**, 4798-4805.

**Storz, P., Doppler, H. and Toker, A.** (2004). Protein kinase Cdelta selectively regulates

protein kinase D-dependent activation of NF-kappaB in oxidative stress signaling. *Mol. Cell. Biol*. **24**, 2614-2626.

**Stumptner, C., Fuchsbichler, A., Heid, H., Zatloukal, K. and Denk, H.** (2002). Mallory body--a disease-associated type of sequestosome. *Hepatology* **35**, 1053-1062.

**Takaesu, G., Ninomiya-Tsuji, J., Kishida, S., Li, X., Stark, G. R. and Matsumoto, K.** (2001). Interleukin-1 (IL-1) receptor-associated kinase leads to activation of TAK1 by inducing TAB2 translocation in the IL-1 signaling pathway. *Mol. Cell. Biol*. **21**, 2475-2484.

**Takatsuna, H., Kato, H., Gohda, J., Akiyama, T., Moriya, A., Okamoto, Y., Yamagata, Y., Otsuka, M., Umezawa, K., Semba, K. et al.** (2003). Identification of TIFA as an adapter protein that links tumor necrosis factor receptor-associated factor 6 (TRAF6) to interleukin-1 (IL-1) receptor-associated kinase-1 (IRAK-1) in IL-1 receptor signaling. *J. Biol. Chem*. **278**, 12144-12150.

**Trompouki, E., Hatzivassiliou, E., Tsichritzis, T., Farmer, H., Ashworth, A. and Mosialos, G.** (2003). CYLD is a deubiquitinating enzyme that negatively regulates NF-kappaB activation by TNFR family members. *Nature* **424**, 793-796.

**Vidalain, P. O., Azocar, O., Servet-Delprat, C., Rabourdin-Combe, C., Gerlier, D. and Manie, S.** (2000). CD40 signaling in human dendritic cells is initiated within membrane rafts. *EMBO J*. **19**, 3304-3313.

**Weernink, P. A. O., Schmidt, M. and Jakobs, K. H.** (2004). Regulation and cellular roles of phosphoinositide 5-kinases. *Eur. J. Pharmacol.* **500**, 87-99.

**Wong, B. R., Besser, D., Kim, N., Arron, J. R., Vologodskaia, M., Hanafusa, H. and Choi, Y.** (1999). TRANCE, a TNF family member, activates Akt/PKB through a signaling complex involving TRAF6 and c-Src. *Mol. Cell* **4**, 1041-1049.

**Wooff, J., Pastushok, L., Hanna, M., Fu, Y. and Xiao, W.** (2004). The TRAF6 RING finger domain mediates physical interaction with Ubc13. *FEBS Lett*. **566**, 229-233.

**Wooten, M. W., Seibenhener, M. L., Mamidipudi, V., Diaz-Meco, M. T., Barker, P. A. and Moscat, J.** (2001). The atypical protein kinase C-interacting protein p62 is a scaffold for NF-kappaB activation by nerve growth factor. *J. Biol. Chem*. **276**, 7709-7712.

**Wooten, M. W., Geetha, T., Seibenhener, M. L., Lewis, M. S., Babu, J. R., Diaz-Meco, M. T. and Moscat, J.** (2005). The p62 scaffold regulates NGF-induced NF-kappa B activation by influencing TRAF6 polyubiquitination. *J. Biol. Chem.* **280**, 35625-35629.

**Xing, L., Venegas, A. M., Chen, A., Garrett-Beal, L., Boyce, B. F., Varmus, H. E. and Schwartzberg, P. L.** (2001). Genetic evidence for a role for Src family kinases in TNF family receptor signaling and cell survival. *Genes Dev*. **15**, 241-253.

**Yamin, T.-T. and Miller, D. K.** (1997). The interleukin-1 receptor-associated kinase is degraded by proteosomes following its phosphorylation. *J. Biol. Chem.* **272**, 21540-21547.

**Yang, K., Zhu, J., Sun, S., Tang, Y., Zhang, B., Diao, L. and Wang, C.** (2004). The coiled-coil domain of TRAF6 is essential for its auto-ubiquitination. *Biochem. Biophys. Res. Commun*. **324**, 432-439.

**Ye, H., Arron, J. R., Lamothe, B., Cirilli, M., Kobayashi, T., Shevde, N. K., Segal, D., Dzivenu, O. K., Vologodskaia, M., Yim, M. et al.** (2002). Distinct molecular mechanism for initiating TRAF6 signalling. *Nature* **418**, 443-447.

**Yoshida, Y., Kumar, A., Koyama, Y., Peng, H., Arman, A., Boch, J. A. and Auron, P. E.** (2004). Interleukin 1 activates STAT3/nuclear factor-kappaB cross-talk via a unique TRAF6- and p65-dependent mechanism. *J. Biol. Chem*. **279**, 1768-1776.

**Zapata, J. M., Pawlowski, K., Haas, E., Ware, C. F., Godzik, A. and Reed, J. C.** (2001). A diverse family of proteins containing tumor necrosis factor receptor-associated factor domains. *J. Biol. Chem*. **276**, 24242-24252.