Thus, some of the same receptors that detect noxious compounds on the tongue to protect animals from ingesting harmful material may also defend human airways. The receptors are positioned on cilia, a location optimal for sampling air and material entering the lung. Moreover, the bitter compound denatonium stimulated ciliary activity, which should hasten elimination of noxious and harmful substances. Substances that activate T2Rs might enter airways through inhalation or aspiration or be generated within the lung by infection. We speculate that this signaling system might also play a role in airway disease. For example, in cystic fibrosis, lungs are commonly infected with *Pseudomonas aeruginosa*. This bacterium produces quorum-sensing molecules that are lactones, which activate some bitter taste receptors (23, 24). Airway T2Rs might also be activated by cigarette smoke, which contains the bitter-tasting compound nicotine (25). In addition, airway cilia are lost in some viral infections and with cigarette smoking (26), which would disrupt this defensive system.

Previous work suggested general roles for the two types of vertebrate cilia—primary cilia are sensory and motile cilia are mechanical. Studies of primary nodal cilia in mouse embryos indicated that the distinction is not absolute; those cilia can exhibit a rotational movement different from the planar beating typical of motile cilia (4, 5). In addition, proteomic studies of *Chlamydomonas flagella* identified numerous proteins involved in signal transduction (27). Our present data indicate that classical motile cilia also have a sensory function.

T2Rs on taste receptor cells, which are not known to have primary cilia, detect bitter ligands and transmit that information to nerves to elicit a response (15, 20). In cells outside the tongue where α-gustducin and T2Rs have been identified—nasal and laryngeal solitary chemosensory cells and intestinal tract enterendocrine cells—signals are transmitted to the associated nerve networks (28–30). In contrast, in airway epithelia, T2Rs enable cell-autonomous detection of a signal followed by a response in the same cell to eliminate harmful substances.

**References and Notes**


**The E3 Ligase TRAF6 Regulates Akt Ubiquitination and Activation**

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Akt signaling plays a central role in many biological functions, such as cell proliferation and apoptosis. Because Akt (also known as protein kinase B) resides primarily in the cytosol, it is not known how these signaling molecules are recruited to the plasma membrane and subsequently activated by growth factor stimuli. We found that the protein kinase Akt undergoes lysine-63 chain ubiquitination, which is important for Akt membrane localization and phosphorylation. TRAF6 was found to be a direct E3 ligase for Akt and was essential for Akt ubiquitination, membrane recruitment, and phosphorylation upon growth-factor stimulation. The human cancer-associated Akt mutant displayed an increase in Akt ubiquitination, in turn contributing to the enhancement of Akt membrane localization and phosphorylation. Thus, Akt ubiquitination is an important step for oncogenic Akt activation.

Protein ubiquitination is an important post-translational modification that regulates various biological functions (1, 2). Although ubiquitination often results in protein degradation, a certain type of ubiquitination is important for signaling activation and protein trafficking (1, 2). Ubiquitination through Lys48 (K48) of the ubiquitin chain generally targets proteins for degradation, whereas ubiquitination through K63 plays a critical role in signaling activation and protein trafficking (1, 2). Akt (also known as protein kinase B) is an important component of cell signaling pathways that regulate cell survival and metabolism. Although membrane recruitment of Akt by growth-factor stimuli is a critical step for Akt phosphorylation, it is not clear how Akt is recruited to the plasma membrane. Because ubiquitination can regulate protein trafficking (1), we tested whether Akt is ubiquitinated in cells. Akt was ubiquitinated in the absence of

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proteasome inhibitor MG132 (fig. S1A). Ubiquitination occurred through K63 but not through K48 (Fig. 1A). Akt ubiquitination could be modified by K11-linked ubiquitin but not through K6 and K48 linkages (fig. S1B).

We screened a panel of E3 ubiquitin ligases for Akt ubiquitination. Although Mdm2 E3 ligase interacts with Akt (4–6), it failed to promote Akt ubiquitination (fig. S2A), as did c-IAP1, c-IAP2, Cbl-b, Itch, Smurf2, and Fbw7 (fig. S2A). Overexpression of c-IAP1 and c-IAP2 reduced Akt ubiquitination (fig. S2, A and B). Because Akt underwent K63 ubiquitination, we focused on TRAF6 and HectH9, two E3 ubiquitin ligases that catalyze K63 ubiquitination and function in Toll-like receptor (TLR) or interleukin-1 (IL-1) signaling and oncogenic activation of Myc, respectively (7–9). TRAF6 promoted Akt ubiquitination, whereas HectH9 did not (Fig. 1B).

Activity of Akt was not required for TRAF6-mediated ubiquitination (fig. S2C). However, TRAF6 E3 ligase activity was required. The TRAF6 C70R→A70 [C70A (J)] mutant, which loses E3 ligase activity (9), had compromised activity (Fig. 1C), which was not due to a defect in Akt interaction (fig. S3A). Although TRAF6 induced Akt ubiquitination, it did not decrease the abundance of Akt (Fig. 1, B and C), suggesting it does not mediate K48 ubiquitination. Indeed, TRAF6 promoted K63 ubiquitination of Akt but not K48 ubiquitination (Fig. 1D). TRAF6, but not TRAF6 C70A, induced Akt ubiquitination in vitro (Fig. 1E and fig. S2D). These results suggest that TRAF6 is an E3 ubiquitin ligase for Akt.

Coimmunoprecipitation experiments revealed that Akt interacted with overexpressed TRAF6 and with TRAF6 C70A (fig. S3A). We detected interaction between endogenous Akt and TRAF6 in cells stimulated with insulin-like growth factor–1 (IGF-1) or IL-1β (fig. S3B), both of which activate Akt signaling. Glutathione-S-transferase (GST)–tagged TRAF6 interacted with Akt directly in vitro (fig. S3C). Ubiquitination of the Akt1 and Akt2 isoforms, but not that of Akt3, was induced by TRAF6 (fig. S4A). Coimmunoprecipitation assay revealed that TRAF6 interacted with all Akt isoforms (fig. S4B).

Overexpression of TRAF6, but not that of TRAF6 C70A, enhanced phosphorylation of Akt at T308 (J) but not at S473, and this enhancement was accompanied by increased Akt activity toward glycogen synthase kinase 3β (GSK3β) (Fig. 1F) (10, 11). A control E3 ligase, HectH9, failed to regulate phosphorylation of Akt (fig. S5). Overexpression of c-IAP1 and c-IAP2, which slightly inhibited ubiquitination of Akt, reduced phosphorylation of Akt on T308 (fig. S2B). Thus, TRAF6 may enhance Akt phosphorylation by promoting Akt ubiquitination.

We compared Akt ubiquitination and phosphorylation in Traf6+/+ and Traf6–/– primary mouse embryonic fibroblasts (MEFs) treated with activators of Akt. Upon IGF-1 treatment, endogenous Akt ubiquitination was reduced in Traf6–/– MEFs compared with that in Traf6+/+ MEFs with TRAF6 or TRAF6 C70A. (D) IB of lysed 293T cells transfected with Akt and TRAF6, along with HA–Ub K48 (K48-only ubiquitin) or HA–Ub K63 (K63-only ubiquitin). IP, immunoprecipitation. (E) GST-Akt-Flag proteins were incubated with adenosine triphosphate, E1, and E2 along with GST–TRAF6 and GST–TRAF6 C70A proteins for in vitro ubiquitination of Akt. (F) WCE from 293T cells transfected with indicated plasmids was collected for IB analysis.

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MEFs (Fig. 2A). Similarly, Akt ubiquitination was induced by 10% fetal bovine serum (FBS) or IL-1β in Traf6+/− MEFs but not in Traf6−/− MEFs (fig. S6A). Consequently, Akt phosphorylation at T308 and S473 after IGF-1 treatment was reduced in Traf6+/− MEFs compared with that in Traf6+/+ MEFs (Fig. 2B). This defect in Akt phosphorylation in Traf6+/− MEFs was correlated with an impairment in phosphorylation of Foxo1 and Foxo3a, two Akt substrates (10, 11). Ubiquitination of TRAF6 was also induced by IGF-1 stimulation (fig. S7A). TRAF6 interacted with IGF-1 receptor (IGF-1R) in serum-free conditions but not after IGF-1 treatment (fig. S7B). Thus, activated IGF-1 receptor may directly engage TRAF6 activation.

Phosphorylation of Akt T308 and S473 was also inhibited in Traf6−/− MEFs treated with 10% FBS (fig. S6B). In contrast, phosphorylation of ERK1 and ERK2 was comparable in wild-type and Traf6−/− MEFs (fig. S6B), suggesting that TRAF6 selectively affects Akt activation. IL-1 and lipopolysaccharide (LPS) activate the IL-1 receptor (IL-1R) and Toll-like receptor 4 (TLR4), respectively. Because TRAF6 is a critical regulator for both IL-1R and TLR4 signaling (8, 9), we tested whether IL-1β–induced and LPS–induced phosphorylation of Akt acts through TRAF6. LPS and IL-1β induced phosphorylation of Akt at T308 in Traf6+/+ MEFs in the presence of 10% FBS; this effect was reduced in Traf6−/− MEFs (fig. S6, C and D). Neither LPS nor IL-1β was sufficient to trigger phosphorylation of Akt at either T308 or S473 in serum-starved conditions (0.1% FBS) in multiple primary cell lines (fig. S8), suggesting that IL-1β and LPS may require cooperation with growth-factor receptor signaling to induce Akt activation. Restoration of TRAF6 expression in Traf6−/− MEFs rescued the defect of Akt phosphorylation in
cells stimulated with IGF-1 or IL-1β, whereas restoration of TRAF6 C70A did not (Fig. 2C and fig. S6E). Thus, TRAF6 is critical for Akt phosphorylation and activation through induction of Akt ubiquitination.

Because Akt influences cell survival and apoptosis (10, 11), we tested whether Traf6 deficiency sensitized cells to apoptosis after serum withdrawal. Apoptosis in Traf6−/− MEFS was higher than in Traf6+/− MEFS in the presence and absence of serum (Fig. 2D and fig. S9). The active, cleaved form of caspase 3, a mediator of apoptosis, was more abundant in Traf6−/− MEFS than in Traf6+/− MEFS (fig. S10A). Restoration of TRAF6, but not TRAF6 C70A, rescued Traf6−/− MEFS from apoptosis (Fig. 2E). A constitutively active form of Akt partially rescued cells from apoptosis (Fig. 2E). Thus, other signaling pathways may be also involved. Because apoptosis inducers such as DNA damage agents induce phosphorylation and activation of Akt (12, 13), we tested whether TRAF6 was also involved. Doxorubicin (Dox)- and cisplatin (Cis)-induced phosphorylation of Akt T308 in wild-type MEFS was impaired in Traf6−/− MEFS (fig. S10B). The impairment of Akt phosphorylation was correlated with increased caspase 3 activation in Traf6−/− MEFS (fig. S10C).

TRAF6 is expressed in most mouse tissues (14, 15). We compared Akt activity in skeletal muscle and heart tissues from wild-type and Traf6−/− mice. Steady-state Akt activity in heart muscle, but not skeletal muscle, was lower in Traf6−/− mice than in wild-type mice (fig. S11). Akt activation in animals injected with IGF-1 was impaired in both forms of muscles in Traf6−/− mice (Fig. 2F). These results suggest that TRAF6 plays a critical role in Akt activation in vivo.

Because the pleckstrin homology (PH) domain of Akt is critical for phosphatidylinositol (3,4,5)-trisphosphate (PIP3) lipid binding and protein-protein interaction (16, 17), we analyzed whether the PH domain of Akt influenced TRAF6-mediated ubiquitination of Akt. TRAF6 failed to promote ubiquitination of the Akt mutant devoid of the PH domain (Fig. 3A). Of the six lysine residues within the PH domain of Akt, mutation on either K8 or K14 to arginine (R) most substantially reduced Akt ubiquitination and Akt phosphorylation at T308 and S473 (Fig. 3B). The K8 and K14 residues are well conserved from Drosophila to humans (fig. S12), suggesting that the ubiquitination of Akt may be evolutionarily conserved.

Akt K8R bound effectively to isolated PIP3, but Akt K14R did not (Fig. 3C). The K14 residue lies within the PIP3 lipid-binding pocket (18–20). Overexpression of TRAF6 did not enhance the binding of Akt to PIP3 (Fig. 3C). Thus, Akt ubiquitination by TRAF6 appears not to influence PIP3 lipid binding, and the defect in phosphorylation of Akt K8R is not due to its impairment in PIP3 binding.

A mutation in the PH domain [E17→K17, E17K (3)] of Akt has been identified in human cancer patients, including those with breast cancer (19). This cancer-associated Akt mutant exhibited constitutive Akt phosphorylation at T308 but not at S473 and had greater oncogenic potential. Basal ubiquitination of the E17K mutant was much higher than that of wild-type Akt (Fig. 3D). Overexpression of TRAF6 still increased ubiquitination of this mutant but to a lesser extent than wild-type Akt (Fig. 3D). The E17K mutant displayed higher Akt phosphorylation at T308 but not at S473 (19), and this was not increased by TRAF6 overexpression (Fig. 3D). Ubiquitination of a K8R/E17K Akt mutant in vivo was reduced and correlated with a reduction in phosphorylation of Akt T308 (Fig. 3E). Thus, increased Akt ubiquitination apparently contributes to the hyperactivation of Akt observed in the Akt E17K mutant.

Because K63 ubiquitination regulates protein trafficking, we tested whether TRAF6 influenced membrane recruitment of Akt. Overexpression of TRAF6 increased Akt membrane localization, which correlated with an increase in phosphorylation and ubiquitination of Akt (Fig. 3A). IGF–1-induced Akt membrane localization and T308 phosphorylation in wild-type MEFS was abolished in Traf6−/− MEFS (Fig. 4B).

The PIP3 binding was calculated as the ratio between amounts of Akt bound with PIP3 beads and total amounts of Akt. (D and E) IBs of lysed 293T cells transfected with indicated plasmids.
Thus, TRAF6 is required for Akt membrane recruitment and phosphorylation upon IGF-1 stimulation.

We also compared the membrane recruitment of wild-type Akt and Akt mutants (K8R and K14R), which are defective in Akt ubiquitination. Membrane recruitment of Akt K8R and K14R upon IGF-1 treatment was reduced (Fig. 4C and fig. S13). The Akt E17K mutant localized to the membrane, even without IGF-1 stimulation, although IGF-1 stimulation further increased membrane recruitment (Fig. 4C and fig. S13). This suggests that Akt ubiquitination contributes to membrane recruitment and phosphorylation of Akt.

Because deregulated Akt can contribute to cancer development (21, 22), we depleted TRAF6 in PC-3 prostate cancer cells by using short hairpin RNAs (shRNAs). Depletion of TRAF6 reduced Akt phosphorylation at T308 and S473 (Fig. 4D). In cells treated with IGF-1, Akt phosphorylation in TRAF6 knockdown cells was impaired (Fig. 4E). In xenograft tumor models, the two stable TRAF6 knockdown cells had lower tumorigenic potential than control cells (Fig. 4F and fig. S14). Thus, TRAF6 appears to influence tumorigenesis in this model.

TRAF6 has an important role in TLR signaling and the innate immune response. Our results expand its known function to include the survival and oncogenic signaling pathways (fig. S15). We suggest that TRAF6 may be a previously uncharacterized oncogene that may serve as an important therapeutic target for human cancers.

References and Notes
3. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
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Figs. S1 to S15

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Materials and Methods

Mice, cell cultures, and reagents. MEFs from WT and Traf6<sup>−/−</sup> mice were prepared as previously described (1, 2). MEFs, NIH3T3, COS-1, and 293T cells were cultured in DMEM containing 10% FBS. pcDNA6-HA-Akt1 K8R, pcDNA6-HA-Akt1 K14R, pcDNA6-HA-Akt1 K20R, pcDNA6-HA-Akt1 K30R, pcDNA6-HA-Akt1 K39R, pcDNA6-HA-Akt1 K64R, pcDNA6-HA-Akt1 E17K, and pcDNA6-HA-Akt1 K88/R17K constructs were generated using a site-directed mutagenesis kit (Stratagene) according to the manufacturer’s standard procedures with pcDNA6-HA-Akt1 as the template. Flag-Traf6, Flag-Traf6 C70A, pGEX-4X1-Traf6, and pGEX-4X1-Traf6 C70A constructs have been described previously (3). Akt KD (Akt K179A) and Mdm2 have been described previously (4). (His)6-ubiquitin and HA-Akt1 were gifts from Drs. D. Bohmann and M.C. Hung, respectively. Flag-SmurF2 and Flag-FBW7 were obtained from Drs. J.L. Wrana and M.H. Lee, respectively. Flag-c-IAP1 and Flag-c-IAP2 were obtained from Dr. X. Yang. Myc-ITCH and HA-Cbl-b constructs were from Drs. A.M. Weissman and S. Lipkowitz, respectively. HectH9, (His)6-ubiquitin K6R, (His)6-ubiquitin K11R, (His)6-ubiquitin K48R, and (His)6-ubiquitin K63R were from Dr. M. Eilers. IGF-1 was obtained from Calbiochem. IL-1β was obtained from Invitrogen. LPS was purchased from Sigma. Doxorubicin and cisplatin were purchased from MP Biomedicals and ALEXIS Biochemicals, respectively.

Immunoprecipitation (IP), immunoblotting (IB), and immunofluorescence. IP, IB, and immunofluorescence were performed essentially as described elsewhere (1, 4). For protein-protein interactions, cells were lysed by E1A lysis buffer [250 mM NaCl, 50 mM HEPES (pH 7.5), 0.1% NP-40, 5 mM EDTA, protease inhibitor cocktail (Roche)]. The following antibodies were used for IP and IB: anti-Traf6 antibody (IP: 1:100; IB: 1:1000, Santa Cruz), anti-ubiquitin antibody (IB: 1:1000, Santa Cruz), anti-N-cadherin (IB: 1:1000, Santa Cruz), anti-Akt1 antibody (IP: 1:200; IB: 1:1000, Cell Signaling), anti-phospho (S473)-Akt antibody (IB: 1:2000, Cell Signaling), anti-phospho (T308)-Akt (IB: 1:1000, Cell Signaling), cleaved caspase-3 (IB: 1:500, Cell Signaling), anti-phospho-Akt (T24)/Foxo3α (T32) antibody (IB: 1:1000, Cell Signaling), anti-phospho-ERK antibody (IB: 1:2000, Cell Signaling), anti-ERK antibody (IB: 1:2000, Cell Signaling), anti-phospho (S1462)-TSC2 antibody (IB: 1:1000, Cell Signaling), anti-TSC2 antibody (IB: 1:1000, Cell Signaling), anti-phospho (S9)-GSK3β antibody (IB: 1:1000, Cell Signaling), Anti-GSK3β antibody (IB: 1:3000, BD Transduction Lab), anti-Xpress antibody (IP: 1:500; IB: 1:5000, Invitrogen), anti-HA antibody (IB: 1:1000, Covance, Upstate), anti-α-tubulin antibody (IB: 1:1000, Sigma), anti-β-actin antibody (IB: 1:1000, Sigma), and anti-Flag antibody (M2, IP: 1:200; IB: 1:3000, Sigma).

GST pulldown assays. For in vitro Traf6 and Akt1 interaction, GST, GST-Traf6, and GST-Traf6 C70A proteins purified from the bacterial lysates of BL21 competent cells transformed with pGEX-4X1, pGEX-4X1-Traf6, and pGEX-4X1-Traf6 C70A using glutathione-agarose beads according to the manufacturer’s standard procedures. The GST, GST-Traf6, and GST-Traf6 C70A proteins bound to glutathione Sepharose beads (Amersham Biosciences) were then incubated with the in vitro translated [35S]-Akt1 for 4 h at 4°C in the interaction buffer [20 mM HEPES (pH 7.9), 150 mM KCl, 5 mM EDTA, 0.5 mM diithiothreitol (DTT), 0.1% (v/v) NP-40, 0.1% (w/v) BSA, 1 mM PMSF, and 10% glycerol], washed with NETN buffer [20 mM Tris (pH 8.0), 100 mM NaCl, 6 mM MgCl2, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM DTT, 8% glycerol, and 1 mM PMSF] 4 times, and subjected to 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography.

Ubiquitination assay. In vivo ubiquitination assays were performed as described elsewhere (5). In brief, 293T cells were transfected with the indicated plasmids for 48 h and lysed by denatured buffer (6 M guanidine-HCl, 0.1 M Na2HPO4/NaH2PO4, 10 mM imidazole), followed by nickel bead purification and Immunoblot analysis. In vitro ubiquitination assays using GST-Traf6 and GST-Akt-Flag were performed as described elsewhere (3). Purified GST-Traf6 and GST-Akt-Flag were incubated for 3 h at 37°C in 20 μl of reaction buffer [20 mM Hepes (pH 7.4), 10 mM MgCl2, 1 mM DTT, 59 μM ubiquitin, 50 mM E1, 850 nM of Ubc13/Uev1a, 1 mM ATP, 30 μM creatine phosphate, and 1 U of creatine kinase]. After incubation, the beads were washed five times in Buffer A [20 mM Tris (pH 7.4), 250 mM NaCl, 1 mM DTT, 1 mM sodium orthovanadate, 2 mM EDTA, and 1% Triton X-100] and two times in low-salt buffer [20 mM Tris (pH 7.4), 25 mM NaCl, and 1 mM DTT]. Beads were then resuspended in 1% SDS (in water) and boiled for 10 min. Dissociated proteins were diluted with 2×600 μl Buffer A, and the supernatant fluid was preclotted with Protein A/G beads for 1 h, and immunoprecipitated overnight with 1 μg anti-Flag antibody, after which Protein A/G beads were added for an additional 1 h. Beads were washed 4 times with Buffer A and 2 times with low-salt buffer. Proteins were eluted in SDS-sample buffer, subjected to SDS-PAGE, transferred to nitrocellulose membrane, and immunoblotted with anti-ubiquitin.
**PIP3 phospholipid binding.** 293T cells transfected with WT HA-Akt and various HA-Akt mutants for 48 h were lysed by E1A buffer, and the cell lysates were incubated with control beads or PIP3 beads (Enchelon) overnight. The beads were washed four times with E1A buffer and subjected to Immunoblot analysis.

**Membrane fractionation.** MEFs were serum-starved for 48 h in DMEM containing 0.1% FBS, and cytosolic and membrane fractions were prepared using the ProteoExtract kit (Calbiochem) according to the manufacturers’ standard procedures.

**Cell apoptosis assay.** WT and Traf6−/− MEFs were serum-starved (0.1% FBS) or cultured in 10% FBS for 2 days, and cells were collected and labeled with Annexin-V-FITC, followed by flow cytometry analysis.

**Viral infection and in vivo tumorigenesis assay.** For retroviral infection, retroviral plasmid PMX, PMX-TRAF6, and PMX-TRAF6 C70A were transfected into Phoenix packing cells for 2 days, and the virus-containing medium was harvested and used to infect primary WT and Traf6−/− MEFs. For lentiviral short hairpin RNA (shRNA) infection, 293T cells were cotransfected TRAF6 or GFP control shRNA with packing plasmids (deltaVPR8.9) and envelope plasmid (VSV-G) using Lipofectamine 2000 reagent according to the manufacturer’s instructions. TRAF6-lentiviral shRNA-1 (5'-GCCACGGGAAATATGTAATATC-3'), TRAF6-lentiviral shRNA-2 (5'-CGAAAGAGATAATGGATGCCAAC-3'), control shRNA (5'-GCAAGCTGACCCTGAAGTTC-3') were transfected with packing plasmids into 293T cells for 2 days, and virus particles containing TRAF6 or control shRNAs were used to infect PC-3 cells. All the infected cells were cultured in medium containing 2 µg/ml puromycin for 4 days. For in vivo tumorigenesis assays, PC-3 stable cells (2×10^6) mixed with matrigel (1:1) were subcutaneously injected into the left flank of 6-week-old athymic nude mice. Tumor size was measured weekly using a caliper, and tumor volume was determined by using the standard formula: \( L \times W^2 \times 0.52 \), where \( L \) is the longest diameter and \( W \) is the shortest diameter.

**Assessing Akt activity in vivo in mice tissues.** Heart and skeletal tissues isolated from 2-week-old WT and Traf6−/− mice (n=5) were lysed in RIPA buffer, and lysates were used to determine Akt activity using the Akt kinase assay kit (Cell Signaling) according to the manufacturer’s standard procedures. For IGF-1 injection, 2 week-old WT and Traf6−/− mice (n=4) were starved for 6 h and intraperitoneally injected with IGF-1 (0.5 mg/kg) for various times. Skeletal and heart muscles were then isolated for the in vitro Akt kinase assay.

**Fig. S1.** Akt undergoes K11 and K63 ubiquitination. (A) Immunoblot (IB) of lysed 293T cells, transfected with HA-Akt and His-Ub constructs for 48 h. (B) 293T cells transfected with Akt along with His-Ub, His-Ub K6R, His-Ub K11R, His-Ub K48R, or His-Ub K63R constructs were lysed for in vivo ubiquitination.

**Fig. S2.** c-IAP-1 and c-IAP-2 reduces Akt ubiquitination and T308 phosphorylation. (A) Immunoblot of lysed 293T cells transfected with HA-Akt, His-Ub, along with various E3 ligases. (B) 293T cells transfected with various plasmids were lysed for in vivo ubiquitination and immunoblot analysis. (C) 293T cells transfected with His-Ub and TRAF6, along with the constitutively active Akt (HA-Akt CA) or kinase dead Akt (HA-Akt KD), were lysed for in vivo ubiquitination. (D) GST-TRAF6 or GST-TRAF6 C70A proteins expressed in bacteria were run on SDS-PAGE, followed by coomassie blue staining.

**Fig. S3.** TRAF6 directly interacts with Akt in vitro and in vivo. (A) 293T cells were transfected with HA-Akt and Flag-TRAF6 or Flag-TRAF6 C70A as indicated for 48 h and lysed for immunoprecipitation with Flag antibody, followed by immunoblot analysis. (B) MEFs cells were serum-starved for 1 day and treated with 100 ng/ml IGF-1 or 20 ng/ml IL-1β for 30 min, and total cell lysates were immunoprecipitated with Akt antibody, followed by Immunoblot analysis. (C) GST-TRAF6 C70A was incubated with in vitro translated [S^35]-Akt, washed, and subjected to SDS-PAGE, followed by autoradiography.

**Fig. S4.** TRAF6 interacts with Akt isoforms and promotes their ubiquitination. (A, B) 293T cells transfected with indicated plasmids and harvested for in vivo ubiquitination (A) and coimmunoprecipitation (B) assays.

**Fig. S5.** TRAF6, but not HectH9, promotes phosphorylation of Akt T308. WCE from 293T cells
transfected with vector, TRAF6, or HectH9 for 48 h were collected for immunoblot analysis.

**Fig. S6.** TRAF6 is required for ubiquitination and phosphorylation of Akt. (A) Traf6^+/+ and Traf6^-^- MEFs were serum-starved for 1 day and were treated with or without 10% FBS or 0 ng/ml IL-1β for 30 min; WCE were collected for immunoprecipitation with Akt, followed by immunoblot analysis. (B) WT and Traf6^-^- MEFs were serum-starved for 1 day, treated with 10% FBS for various time points, and harvested for immunoblot analysis. (C, D) WT and Traf6^-^- MEFs cultured in 10% FBS were treated with 10 µg/ml LPS (C) or 20 ng/ml IL-1β (D) for various times as indicated and harvested for immunoblot analysis. (E) Primary Traf6^-^- MEFs infected with Mock, TRAF6, or TRAF6 C70A mutant were treated with 20 ng/ml IL-1β at various time points and harvested for immunoblot analysis.

**Fig. S7.** TRAF6 interacts with IGF-1R and its ubiquitination is induced by IGF-1. (A, B) COS-1 and PC-3 cells were serum-starved for 1 day and treated with or without 100 ng/ml of IGF-1; WCE were collected for immunoprecipitation with TRAF6, followed by immunoblot analysis with anti-ubiquitin antibody (A) or anti-IGF-1Rβ antibody (B).

**Fig. S8.** Phosphorylation of Akt is not induced by IL-1β and LPS in serum-starved conditions. Primary MEFs (A), IMR90 (B), or WI38 (C) were serum-starved for 1 day, treated with 10% FBS, 100 ng/ml IGF-1, 10 µg/ml LPS, or 20 ng/ml IL-1β in the absence of 10% FBS for various times as indicated and harvested for immunoblot analysis.

**Fig. S9.** TRAF6 regulates cell survival. WT and Traf6^-^- MEFs were cultured in 10% FBS or serum-starved for 2 days, and cell apoptosis was determined by Annexin-V staining, followed by flow cytometry analysis.

**Fig S10.** TRAF6 is required for phosphorylation and activation of Akt upon stimulation with DNA damage agents. (A) WT and Traf6^-^- MEFs were serum-starved for various times and harvested for Immunoblot analysis. (B, C) WT and Traf6^-^- MEFs were treated with Dox and Cis for various times and harvested for immunoblot analysis.

**Fig. S11.** TRAF6 regulates steady-state Akt activity in vivo. Heart and skeletal muscle isolated from WT and Traf6^-^- mice (n=5) was lysed and subjected to an in vitro Akt kinase assay and immunoblot analysis. The graph (lower panel) represents the averaged Akt activity from total 5 mice. Results are presented as mean values ± S.D. *p<0.05, using Student’s t-test.

**Fig. S12.** Conservation of the K8 and K14 residues within the Akt/PKB PH domain between the species.

**Fig. S13.** The Akt K8R and Akt K14R mutants display a defect in membrane recruitment of Akt. (A) NIH3T3 cells transfected with HA-Akt or various Akt mutants for 24 h were serum-starved with 0.1% FBS for 1 day, treated with 100 ng/ml of IGF-1 for 15 min, and fixed for immunofluorescence. The scale bar represents 20 µm. (B) NIH3T3 cells transfected with HA-Akt or various Akt mutants for 24 h were serum-starved with 0.1% FBS for 1 day, treated with 100 ng/ml of IGF-1 for 15 min, and fixed for immunofluorescence. The Arrow indicates the membrane localization of Akt. The scale bar represents 10 µm. (C) Quantification of the experiments shown in (A) A total of 100-200 cells were scored, and a representative result is shown from two independent experiments. Results are presented as mean values ± S.D. *p<0.05, **p<0.001 using Student’s t-test. (D) COS-1 cells were transfected with indicated plasmids, serum-starved, and treated with IGF-1 for 15 min, and the cytosolic fractions were isolated for immunoblot analysis.

**Fig. S14.** TRAF6 silencing inhibits tumorigenic potential of prostate cancer cells. PC-3 cells silenced with control or TRAF6 shRNAs were injected into nude mice (n=6 for each group) and monitored for tumorigenesis. Pictures were taken 3 weeks after the injection.

**Fig. S15.** The working model for Akt activation. IGF-1 engagement to its membrane receptor, IGF-1R, and induces TRAF6 activation. Activated TRAF6 then triggers ubiquitination of Akt, which is prerequisite to membrane recruitment of Akt, where Akt can be anchored in the membrane by binding to the PIP3, followed by phosphorylation of Akt at T308 and S473 induced by PDK1 and mTORC2 (mTOR complex 2), respectively.

**References.**
Supplemental S3

A

IP: Flag

IB: HA

HA-Akt

IgG

IB: Flag

TRAF6

WCE

HA-Akt

TRAF6

HA-Akt

Flag-TRAF6

WT

C70A

WT

C70A

B

IP: IgG

TRAF6

- - IGF-1 IL-1

IB: Akt

Akt

IB: TRAF6

TRAF6

WCE

Akt

TRAF6

C

Input GST GST-TRAF6

35S-Akt

Ponceau S

GST-TRAF6

GST
### Supplemental S4

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- **p-Akt (T308)**
- **p-Akt (S473)**
- **HA-Akt**

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Supplemental S8

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Supplemental S9

The diagram shows flow cytometry histograms comparing the percentage of FITC-Annexin V-positive cells in two different strains of mice, Traf6+/+ and Traf6−/−, under two conditions: + Serum and - Serum.

- **Traf6+/+**
  - + Serum: 7.6%
  - - Serum: 24.3%

- **Traf6−/−**
  - + Serum: 20.3%
  - - Serum: 35.4%
Supplemental S10

A

\[
\begin{array}{cccc}
\text{Traf6}^{+/+} & \text{Traf6}^{-/-} \\
0 & 15 & 30 & 60 & 0 & 15 & 30 & 60 \\
\end{array}
\]

(hrs)

\[
\begin{array}{cc}
\text{Active-Caspase-3} \\
\text{\beta-actin} \\
\end{array}
\]

B

\[
\begin{array}{cccc}
\text{Traf6}^{+/+} & \text{Traf6}^{-/-} & \text{Traf6}^{+/+} & \text{Traf6}^{-/-} \\
0 & 15 & 30 & 60 & 0 & 15 & 30 & 60 & 0 & 15 & 30 & 60 & 0 & 15 & 30 & 60 \\
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\]

(min)

\[
\begin{array}{cc}
p-Akt (T308) & p-Akt (S473) \\
Akt & p-TSC2 \\
TSC2 & p-Foxo3a \\
p-Foxo1 & \text{Foxo1} \\
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C

\[
\begin{array}{cccc}
\text{Traf6}^{+/+} & \text{Traf6}^{-/-} & \text{Traf6}^{+/+} & \text{Traf6}^{-/-} \\
0 & 24 & 48 & 0 & 24 & 48 & 0 & 24 & 48 & 0 & 24 & 48 & 0 & 24 & 48 \\
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\]

(hrs)

\[
\begin{array}{cc}
pAkt (T308) & pAkt (S473) \\
Akt & Active-Caspase-3 \\
\beta-actin \\
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\]

\[\text{Dox} \quad \text{Cis}\]
Supplemental S11

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- Heart

- Muscle

Akt Activity

*P<0.05

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Supplemental S13

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Supplemental S13

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[Images of cellular distributions for different conditions]
Supplemental S13

C

Akt with membrane localization (%)

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**P<0.001**  
*P=0.009  
**P=0.003  
**P=0.001

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IGF-1 binds to IGF-1R, activating PI3K and leading to PIP3 production. PIP3 promotes PH domain of Akt, leading to Akt activation. Akt phosphorylates T308 and S473, downstream of PDK1, activating Akt signaling pathways.

TRAF6 is involved in the ubiquitination of Akt, facilitating its membrane translocation and inactivation.