SUPPLEMENTAL INFORMATION

TIPE3 Is The Transfer Protein Of Lipid Second Messengers That Promote Cancer

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SUPPLEMENTAL DATA

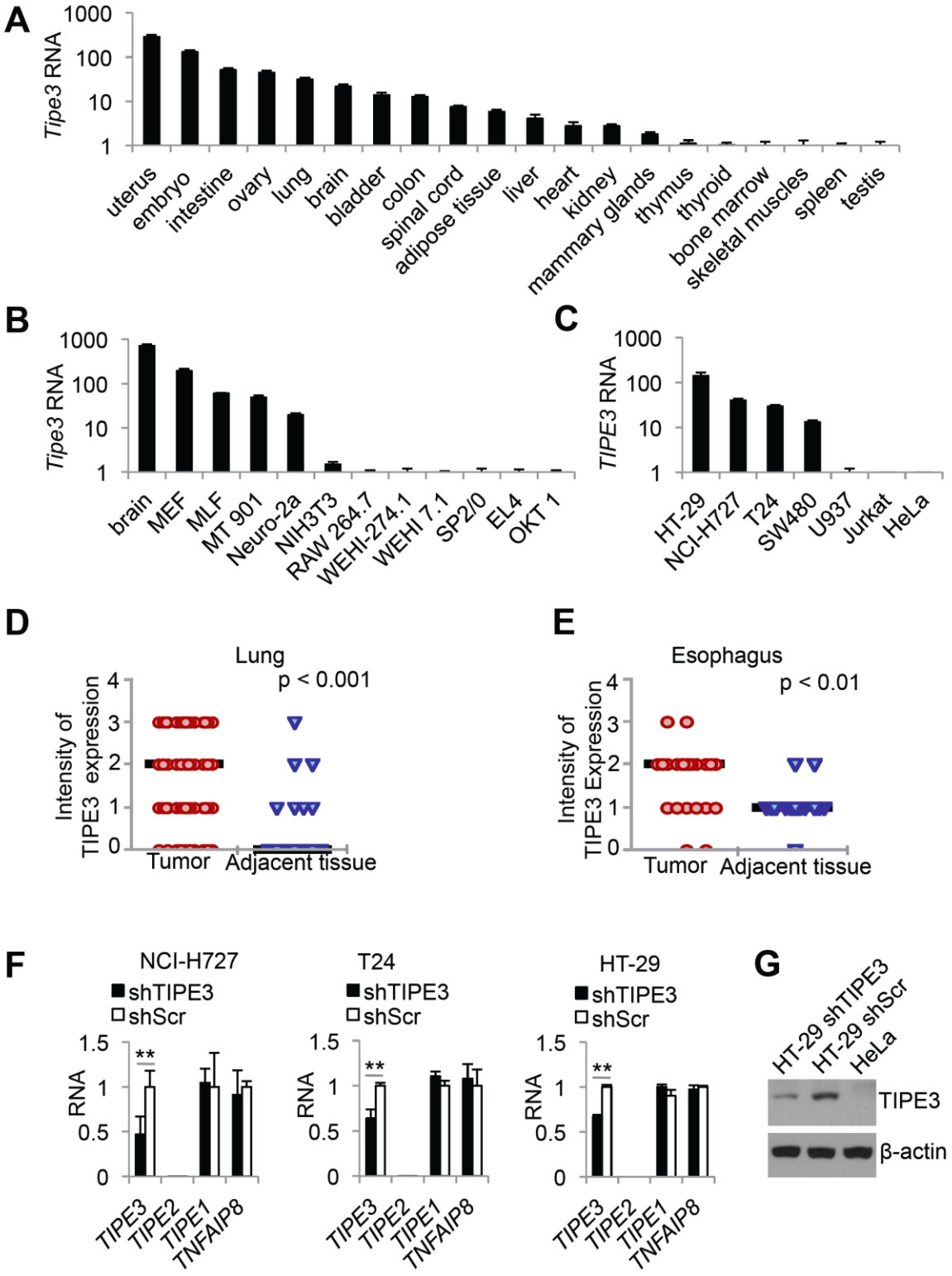


Figure S1, related to Figure 1. TIPE3 expression and TIPE3 knockdown
(A-C) Real-time PCR analyses of TIPE3 expression in murine organs, tissues, or cells (A and B)

and human cell lines (C). The expression levels of TIPE3 in bone marrow (A), RAW 264.7 cells (B), and U937 cells were set as 1. (D and E) Quantitation of the intensity of TIPE3 expression in tumors and adjacent tissues of 60 patients with lung cancer (D) and 24 patients with esophageal cancer (E) was performed as described in Supplemental Experimental Procedures. Each data point represents a TIPE3 expression intensity score of patient's tumor or adjacent tissue. Lines represent medians. (F) Real-time PCR analyses of TIPE3, TIPE2, TIPE1, and TNFAIP8 expression in NCI-H727, T24, and HT-29 cells stably expressing either shTIPE3 or shScr. Levels of TIPE3, TIPE2, TIPE1, and TNFAIP8 expression in NCI-H727, T24, and HT-29 cells stably expressing shScr were set as 1. (G) Whole cell lysates were prepared from HT-29 cells stably expressing either shTIPE3 or shScr, or HeLa cells. Western blot was performed with anti-TIPE3 TH-domain antibody and anti-β-actin antibody. *MLF*, murine lung fibroblasts. Values represent means ± SD, * $p < 0.05$, and ** $p < 0.01$. The experiments were performed in duplicate and repeated at least three times with similar results.

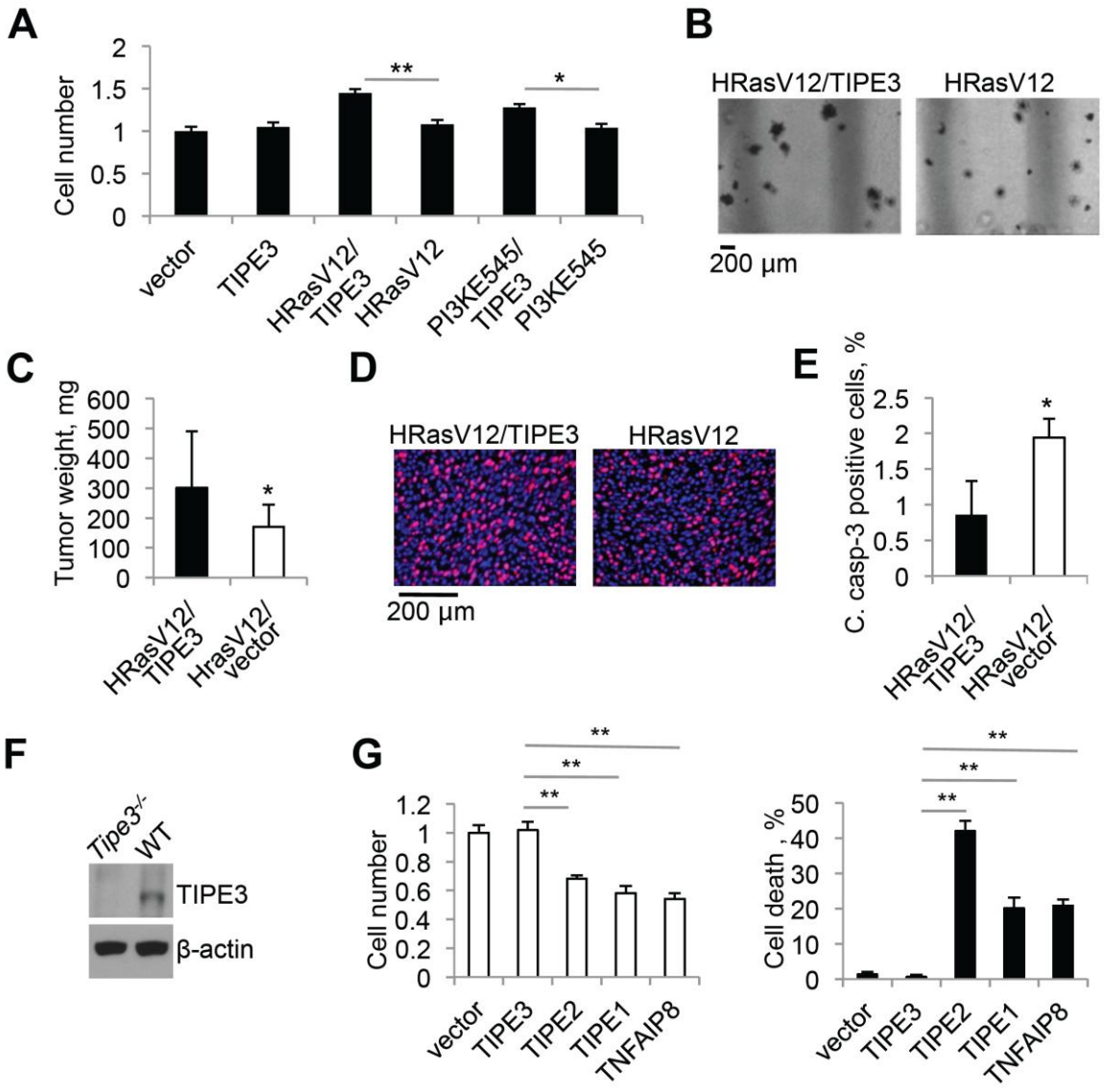


Figure S2, related to Figure 2. TIPE3 promotes tumorigenesis *in vitro* and *in vivo*

(A) 293T cells were transfected with empty vector, HRasV12, PI3KE545, with or without TIPE3-Flag plasmid as indicated and relative cell numbers were determined 32 hr after transfection. Number of 293T cells transfected with empty vector was set as 1. (B) Soft agar colony forming abilities of NIH3T3-HRasV12 cells stably transfected with either TIPE3-Flag or empty vector. (C-E) Tumors generated as described in Figure 2 were weighed and compared (C) Tumor sections from mice that received Brdu injection 2 hr before sacrifice were stained for Brdu by immunofluorescence histochemistry. Brdu staining was detected using a Cy3-labeled secondary antibody and is shown in pink, whereas DAPI-stained nuclei are shown in blue (D) Immunofluorescence staining was also performed on tumor sections with anti-cleaved caspase-3 antibody. The percentages of cleaved caspase-3-positive cells are shown (E). (F) Lysates were prepared from the uteri of 3 *Tipe3*$^{-/-}$ and 3 wild type (WT) mice. Western blot was performed with anti-TIPE3 TH-domain antibody and anti-β-actin antibody. (G) 293T cells were transfected with equal amounts of the following plasmids as indicated: empty vectors, TIPE3-Flag, TIPE2-Flag, TIPE1-Flag, and TNFAIP8-Flag. Relative cell numbers were determined (left panel, number of 293T cells transfected with empty vector was set as 1), and the degree of cell death

was assessed by trypan blue staining (right panel) 32 hr later. For bar graphs, Y-axis values represent means ± SD, * p<0.05, and ** p <0.01. The experiments were repeated at least three times with similar results.

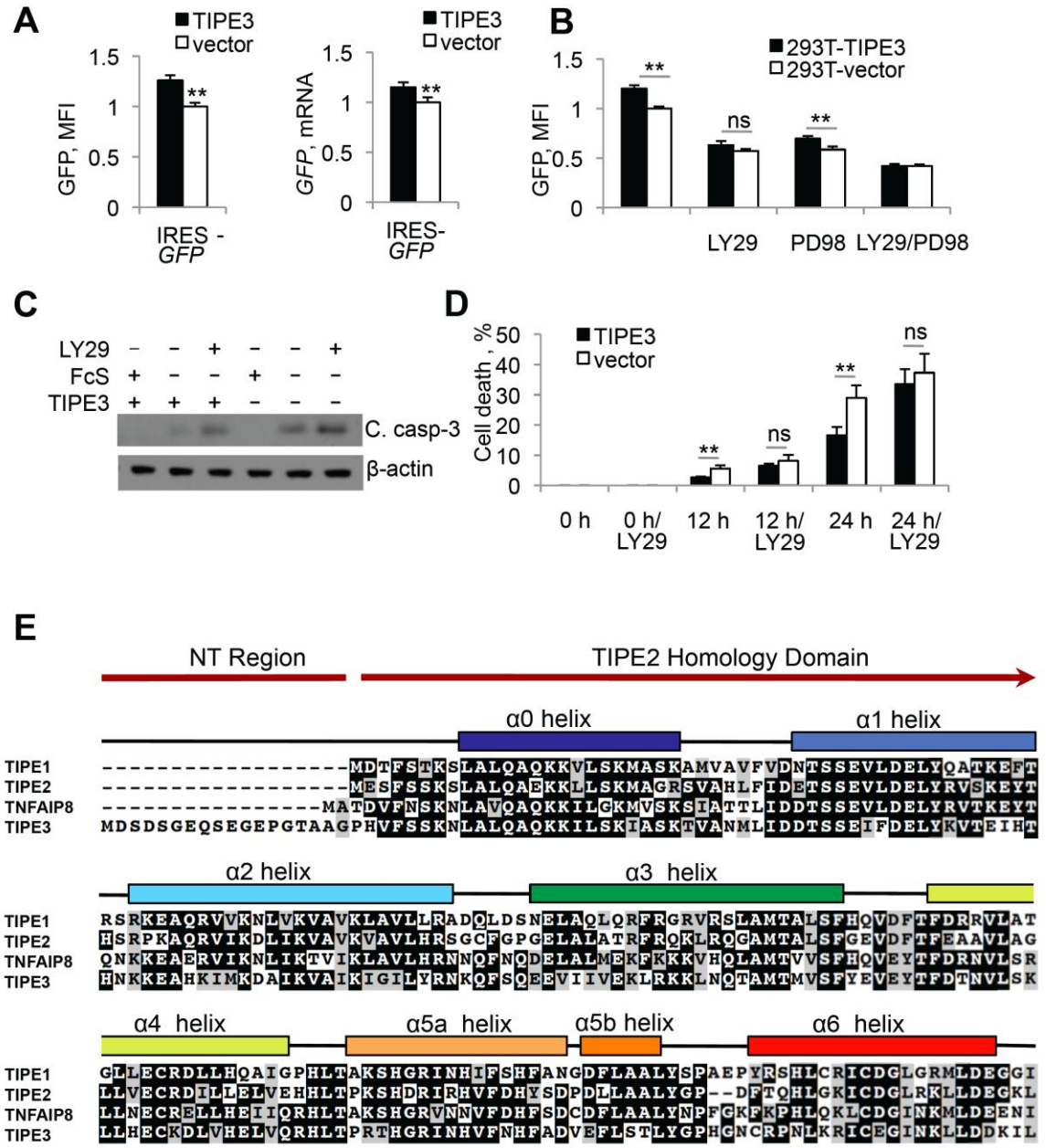


Figure S3, related to Figure 3. The unique roles of TIPE3

(A) 293T cells were co-transfected with pMigR1 (IRES-GFP, for IRES-dependent translation) and either TIPE3-Flag-expressing or empty vector. The relative levels of GFP protein expression 32 hr after the transfection were quantified by measuring the cellular mean fluorescence intensity (MFI) (left panel), and the relative levels of *GFP* mRNA expression were determined by real-time PCR (right panel). Protein and mRNA levels of cells transfected with empty vector were set

as 1. (B) 293T cells that did or did not stably express TIPE3-Flag were transfected with pEGFP-C3 (5'cap-GFP, for cap-dependent translation). After 3.5 hr, cells were cultured with or without LY29 (LY29004) or PD98 (PD98059) inhibitors as indicated. The levels of GFP protein expression were quantified by measuring the cellular MFI 12 hr later. The GFP level of 293T cells stably transfected with empty vector and cultured without inhibitors was set as 1. (C) NIH3T3 cells transfected with either TIPE3-Flag (TIPE3 $^{+}$) or empty vector (TIPE3 $^{-}$) were cultured with or without 10% fetal calf serum (FCS) or LY29 (LY29004) as indicated. After 12 hr, whole cell lysates were prepared and examined by Western blot with anti-cleaved caspase-3 (C. casp-3) and anti-β-actin. (D) NIH3T3 cells transfected with either TIPE3-Flag or empty vector were cultured in serum-free medium with or without LY29 (LY29004) for the indicated times. Cell death was assessed by trypan blue staining. For bar graphs, Y-axis values represent means ± SD; *ns* designates not significant; * $p<0.05$, and ** $p<0.01$; *h* denotes hr. The experiments were repeated three times with similar results. (E) Sequence alignment of murine TNFAIP8, TIPE1, TIPE2, and TIPE3 proteins was generated by CLUSTAL W2. Identical residues are highlighted in black and similar residues are highlighted in gray (generated by the BOXSHADE 3.21).

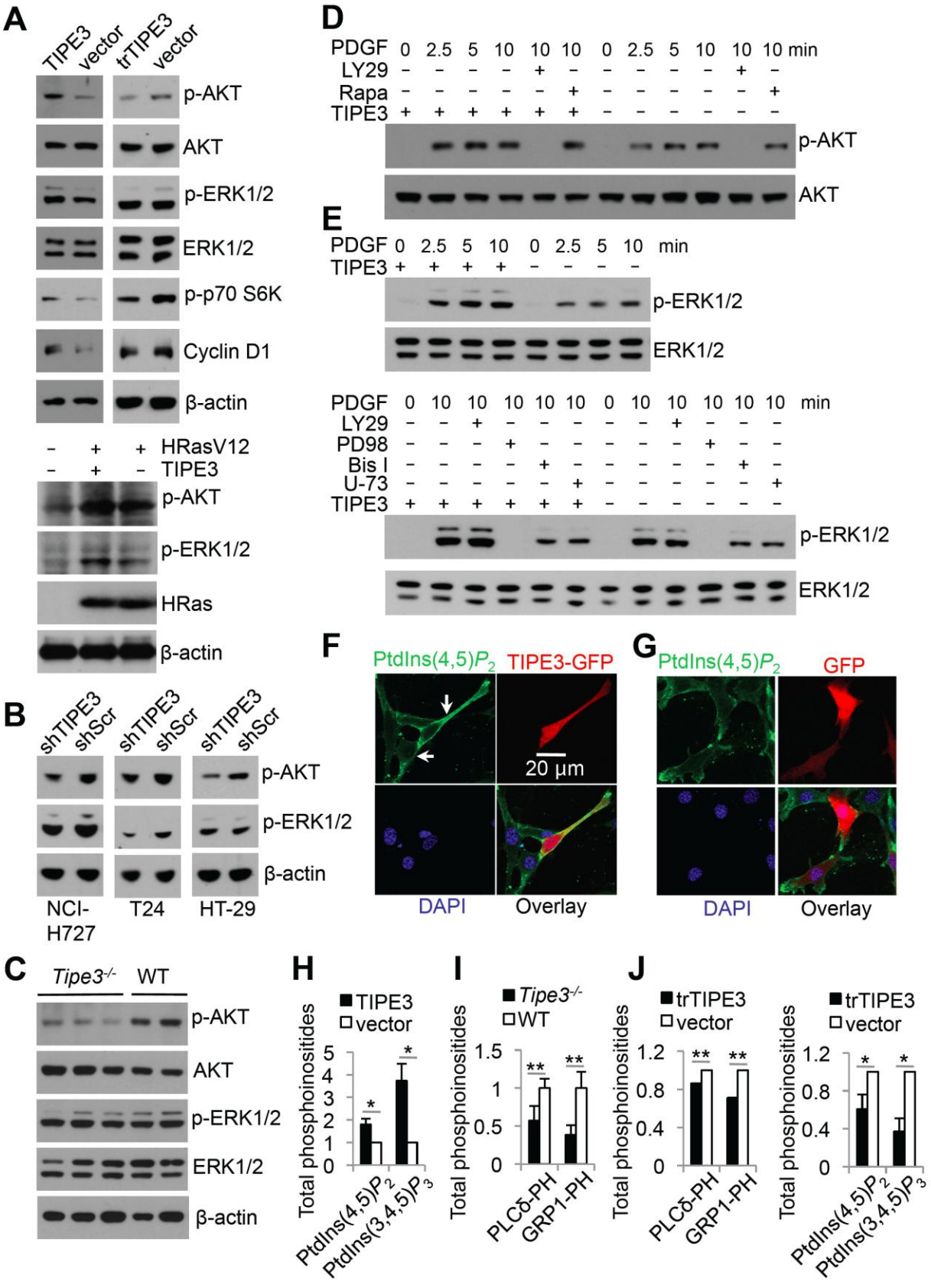
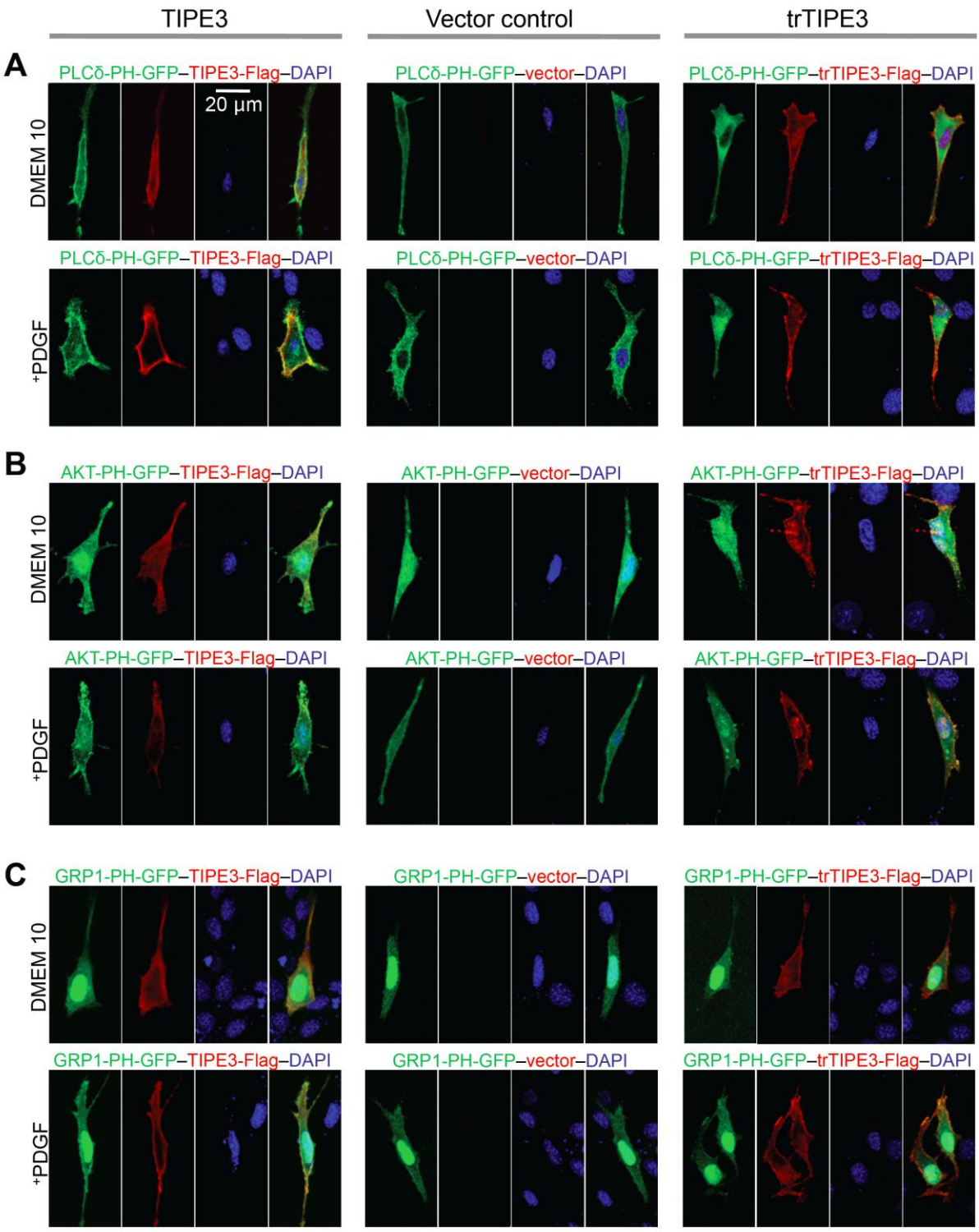


Figure S4, related to Figure 4. TIPE3 promotes the activation of PI3K-AKT and MEK-ERK pathways

(A) Whole cell lysates were prepared from NIH3T3 cells stably transfected with TIPE3-Flag, trTIPE3-Flag, or empty vectors (Top Panel), or TIPE3-Flag and/or HRasV12 vectors (Bottom Panel). Western blot was performed using antibodies against the indicated proteins. (B) Whole cell lysates were prepared from NCI-H727, T24, and HT-29 cells stably expressing either shTIPE3 or shScr. Western blot was performed using antibodies against the indicated proteins. (C) Whole cell lysates were prepared from 3 *Tipe3*$^{-/-}$ and 2 wild type fibrosarcoma cultures established from different 3-methylcholanthrene-induced tumors. Western blot was performed using antibodies against the indicated proteins. (D and E) NIH3T3 cells stably transfected with either TIPE3-Flag (TIPE3 $^{+}$) or empty vectors (TIPE3 $^{-}$) were serum-starved, and then stimulated with 40 ng/ml of PDGF for the indicated times, with or without the following inhibitors: LY29 (LY29004), Rapa (Rapamycin), PD98 (PD98059), Bis I (Bisindolylmaleimide I), or U-73 (U-73122). Western blot was performed using antibodies against the indicated proteins. (F and G) NIH3T3 cells were transfected with TIPE3-GFP (F) or GFP (G) plasmids and analyzed by immunofluorescence confocal microscopy. PtdIns(4,5)P_2 (shown in green) was detected with anti-PtdIns(4,5)P_2 and Alexa Fluor 555-labeled secondary antibody. TIPE3-GFP and GFP are shown in red and DAPI-stained nuclei are shown in blue. For Panel (E), five cells are shown, but only one expresses TIPE3-GFP. For Panel (F), six cells are shown with only three expressing GFP. (H) Cellular levels of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 in NIH3T3 cells stably transfected with either TIPE3-Flag or empty vector were estimated by dot blot with anti-PtdIns(4,5)P_2 and anti-PtdIns(3,4,5)P_3 antibodies. Signals of cells transfected with empty vector were set as 1. (I) Cellular levels of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 in *Tipe3*$^{-/-}$ and wild type fibrosarcoma cells were measured by protein-lipid overlay assay with GST-PLCδ-PH and GST-GRP1-PH proteins. Data are pooled from 3 *Tipe3*$^{-/-}$ and 2 wild type fibrosarcoma cultures established from different 3-methylcholanthrene-induced tumors. Signals of wild type fibrosarcoma cells were set as 1. (J) Cellular levels of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 in NIH3T3 cells stably transfected with either trTIPE3-Flag or empty vector were estimated by protein-lipid overlay assay with GST-PLCδ-PH and GST-GRP1-PH domains (Right Panel) and by dot blot with anti-PtdIns(4,5)P_2 and anti-PtdIns(3,4,5)P_3 antibodies (Left Panel). Signals of cells transfected with empty vector were set as 1. For Panels (H-J) the densitometric quantification of signals was performed using ImageJ software. Y-axis values represent means ± SD, * $p<0.05$, and ** $p< 0.01$. *p-* indicates phosphorylated. The experiments were performed at least three times with similar results.


TIPE3
Vector control
trTIPE3
A
PLCδ-PH-GFP–TIPE3-Flag–DAPI
PLCδ-PH-GFP–vector–DAPI
PLCδ-PH-GFP–trTIPE3-Flag–DAPI
20 μm
DMEM 10
+PDGF
B
AKT-PH-GFP–TIPE3-Flag–DAPI
AKT-PH-GFP–vector–DAPI
AKT-PH-GFP–trTIPE3-Flag–DAPI
C
GRP1-PH-GFP–TIPE3-Flag–DAPI
GRP1-PH-GFP–vector–DAPI
GRP1-PH-GFP–trTIPE3-Flag–DAPI

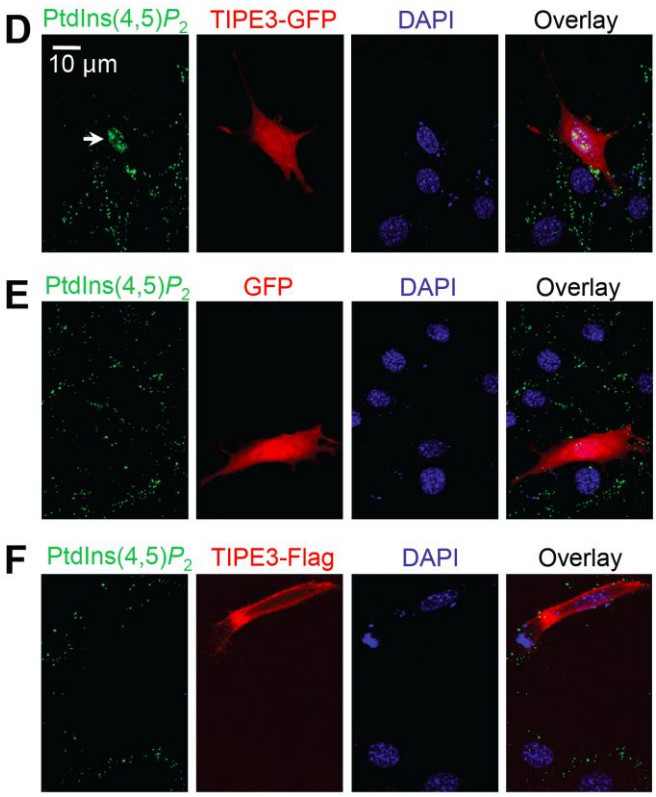


Figure S5, related to Figure 5. TIPE3 regulates phosphoinositide metabolism and signaling
(A-C) Cells were treated and analyzed as described in Figure 4A-4C. GFP signals are shown in green; Flag antibody staining, detected with an Alexa Fluor 555-labeled secondary antibody, is shown in red. For A-C, the experiments were repeated at least three times with similar results. (D-F) NIH3T3 cells were transfected with TIPE3-GFP (D), GFP (E), or TIPE3-Flag (F) plasmids. Thirty-two hr after the transfection, cells were fixed, permeabilized with 0.1% Triton X-100, and analyzed by immunofluorescence confocal microscopy. PtdIns(4,5)P_2 detected with anti-PtdIns(4,5)P_2 and Alexa Fluor 555-labeled secondary antibody is shown in green. The plasma membrane PtdIns(4,5)P_2 is seen as multiple dots due to the loss of lipid bilayer integrity under the permeabilization condition. TIPE3-GFP (D), GFP (E) and TIPE3-Flag detected with anti-Flag Alexa Fluor 488 antibody (F) are shown in red. For Panel D, four cells are shown, but only one expresses TIPE3-GFP. For Panel E, two cells are shown with one expressing GFP. For Panel F, three cells are shown with only one expressing TIPE3-Flag. For D-F, the experiments were repeated at least two times with similar results. For A-F, DAPI-stained nuclei are shown in blue. The last image in each group is an overlay of the other three.

Table S1, related to Figure 6. Crystallographic statistics for the TH-domain of human TIPE3.

Data	TIPE3 (21-204)
Space Group	I222
Cell dimentions a, b, c (Å), α, β, γ (°)	61.73, 87.30, 242.31, 90, 90, 90
Number of molecules in ASU	2
Wavelength (Å)	0.9796
Resolution (Å)	40~2.30 (2.38~2.30)
Rmerge (%)	7.3 (38.2)
I/σ	30.4 (6.6)
Completeness (%)	99.4 (99.9)
Number of measured reflections	199733
Number of unique reflections	29376
Redundancy	6.8 (7.0)
Wilson B factor ($Å^2$)	43.2
R-factor (%)	23.98
Rfree (%)	26.19
Number of atoms	
Protein main chain	1498
Protein side chain	1557
Protein all atoms	3055
Water molecules	137
Other entities	44
All atoms	3236
Average B value ($Å^2$)	
Protein main chain	54.8
Protein side chain	62.9
Protein all atoms	58.9
Water molecules	58.7
Other entities	108.6
All atoms	59.5
Rms deviations from ideal values	
Bonds (Å)	0.010
Angle (°)	1.219
Ramachandran plot statistics (%)	
Most favorable	94.1
Additionally allowed	5.7
Generously allowed	0.3
Disallowed	0

Values for highest resolution shell are shown in parentheses. $R_{merge}=\Sigma_h\Sigma_i|I_{h,i}-I_h|/\Sigma_h\Sigma_i I_{h,i}$, where I_h is the mean intensity of the i observations of symmetry related reflections of h. $R=\Sigma|F_{obs}-F_{calc}|/\Sigma F_{obs}$, where F_{calc} is the calculated protein structure factor from the atomic model (R_{free} was calculated with 5% of the reflections selected).

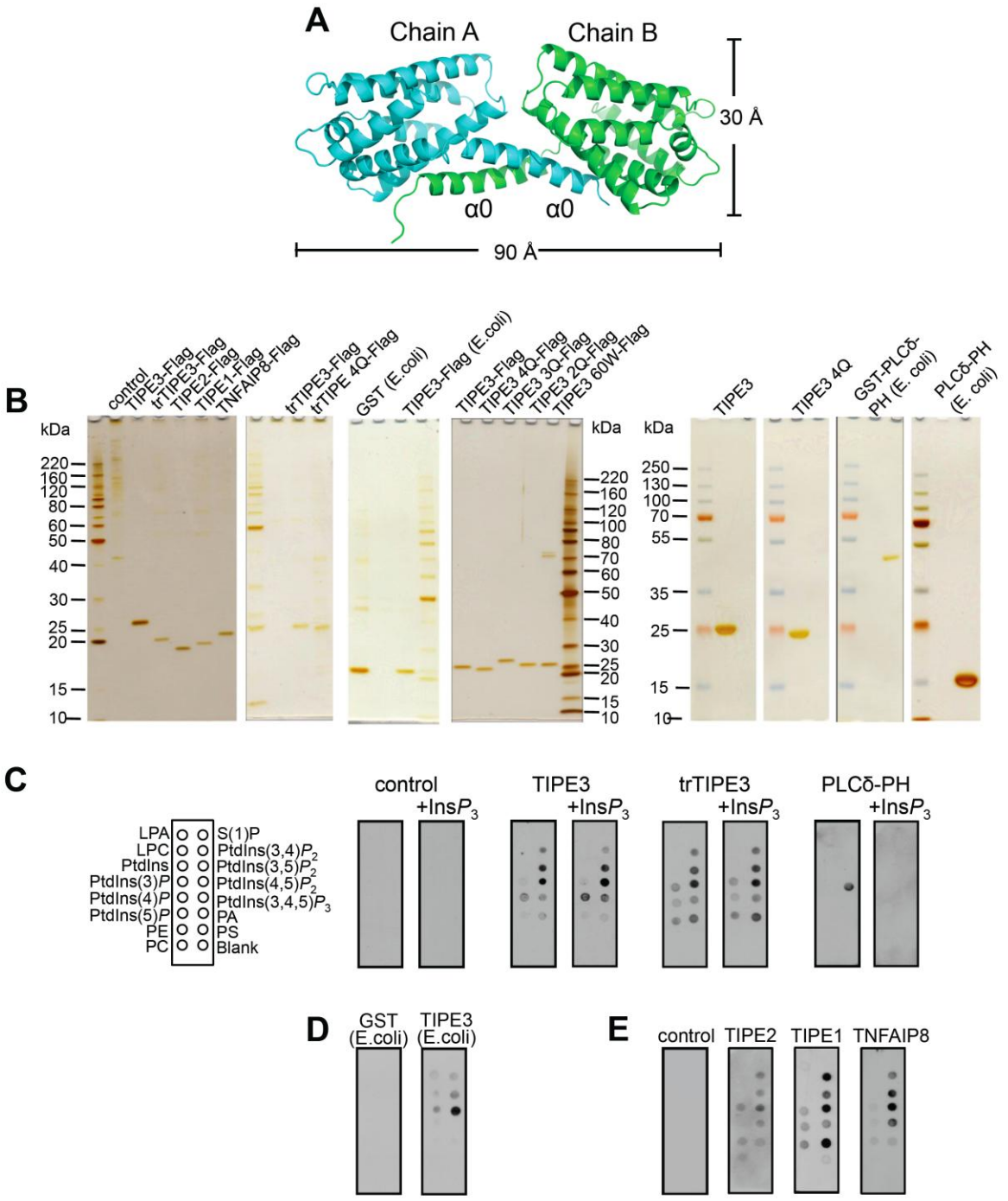


Figure S6, related to Figure 6. TIPE3 binds to phosphoinositides

(A) Cartoon representation of trTIPE3 homodimer formed in the crystal. Chains A (residues 22-204) is shown in cyan and chain B (residues 21-204) is shown in green. (B) TIPE3-Flag, TIPE3 4Q-Flag, TIPE3 3Q-Flag, TIPE3 2Q-Flag, TIPE3 60W-Flag, trTIPE3-Flag, trTIPE3 4Q-Flag, TIPE2-Flag, TIPE1-Flag, TNFAIP8-Flag, TIPE3, and TIPE3 4Q proteins were purified from 293T cells. GST, TIPE3-Flag (*E. coli*), GST-PLCδ-PH, and PLCδ-PH were purified from *Escherichia coli* BL21 cells. Purified proteins were separated by SDS-PAGE and stained with

silver. The HSPA8 and HSPA1A protein bands (identified by mass spectrometry) are also seen in the TIPE3 60W-Flag lane. (C) Binding of TIPE3-Flag, trTIPE3-Flag (purified after overexpression in 293T cells), and GST-PLCδ-PH proteins to lipids spotted on PIP strips, in the presence or absence of inositol-1,4,5-trisphosphate (Ins(1,4,5)P_3), as determined by protein-lipid overlay assay. Control eluate as described in Supplemental Experimental Procedures was used as the control. LPA, lysophosphatidic acid; LPC, lysophosphocholine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; S(1)P, sphingosine-1-phosphate; PA, phosphatidic acid; PS, phosphatidylserine. (D and E) Binding of (D) GST and TIPE3-Flag proteins (purified from *Escherichia coli* BL21 cells) and (E) TIPE2-Flag, TIPE1-Flag and TNFAIP8-Flag proteins (purified from 293T cells) to phosphoinositides spotted on PIP strips as determined by protein-lipid overlay assay.

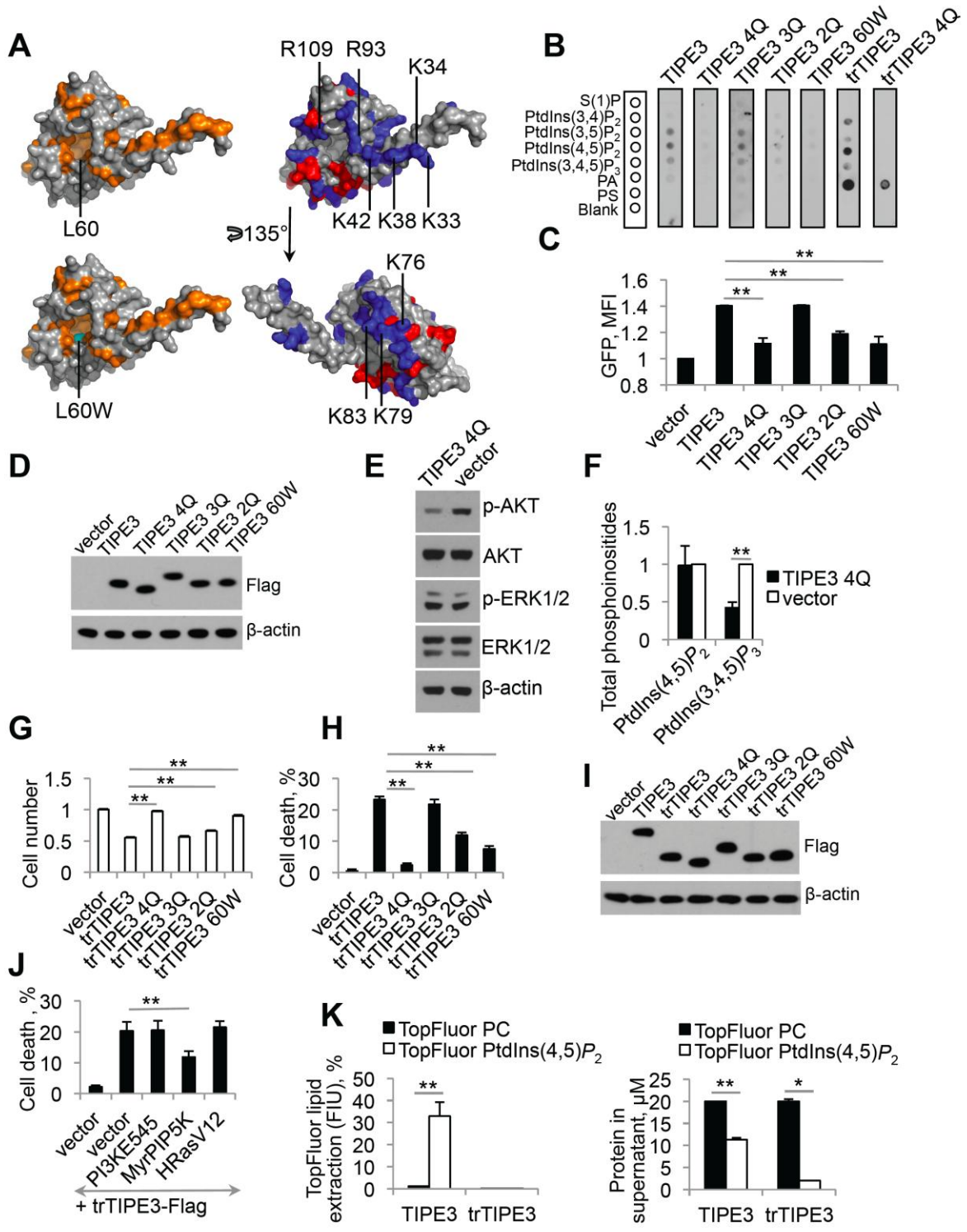


Figure S7, related to Figure 7. TIPE3 binds to phosphoinositides through its TH domain and the binding is essential for its function

(A) The structure models of wild type murine TIPE3 (residues 21-204) and murine TIPE3 mutants (residues 21-204) generated based on human TIPE3 crystal structure using Phyre[2] and PyMOL are shown in surface presentation. Amino acids with hydrophobic side chains are shown in orange, those with positively charged side chains are in blue, and those with negatively

charged side chains are colored red. Tryptophan positioned inside the hydrophobic pocket, which is mutated in TIPE3 60W, as well as several other residues mutated in this study are marked. (B) Binding of recombinant TIPE3-Flag, TIPE3 4Q-Flag, TIPE3 3Q-Flag, TIPE3 2Q-Flag, TIPE3 60W-Flag, trTIPE3-Flag, and trTIPE3 4Q-Flag proteins to lipids spotted on PIP strips as determined by protein-lipid overlay assay. (C) 293T cells were co-transfected with pEGFP-C3 and one of the following plasmids: empty vector, TIPE3-Flag, TIPE3 4Q-Flag, TIPE3 3Q-Flag, TIPE3 2Q-Flag, and TIPE3 60W-Flag. The relative levels of GFP protein expression were quantified by measuring cellular MFI 32 hr after the transfection. GFP level of cells transfected with empty vector was set as 1. (D) 293T cells were co-transfected with pEGFP-C3 and one of the following plasmids: empty vector, TIPE3-Flag, TIPE3 4Q-Flag, TIPE3 3Q-Flag, TIPE3 2Q-Flag, and TIPE3 60W-Flag. The levels of Flag-tagged proteins and actin were determined by Western blot 16 hr after the transfection. (E) Whole cell lysates were prepared from NIH3T3 cells stably transfected with either TIPE3 4Q-Flag or empty vector. Western blot was performed using antibodies against the indicated proteins. (F) Total levels of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 in NIH3T3 cells stably transfected with either TIPE3 4Q-Flag or empty vector were measured by protein-lipid overlay assay with GST-PLCδ-PH and GST-GRP1-PH domains. The densitometric quantification of signals was performed using ImageJ software. Signals of cells transfected with empty vector were set as 1. (G and H) 293T cells were transfected with 4 μg of the following plasmids: empty vector, trTIPE3-Flag, trTIPE3 4Q-Flag, trTIPE3 3Q-Flag, trTIPE3 2Q-Flag, and trTIPE3 60W-Flag. Thirty-two hr after the transfection, cells were stained with trypan blue, and relative cell numbers (G) and percentages of dead cells (H) determined by light microscopy. The number of 293T cells transfected with empty vector was set as 1. (I) 293T cells were transfected with 4 μg of the following plasmids: empty vector, trTIPE3-Flag, trTIPE3 4Q-Flag, trTIPE3 3Q-Flag, trTIPE3 2Q-Flag, or trTIPE3 60W-Flag. The levels of Flag-tagged proteins and actin were determined by Western blot 16 hr after the transfection. (J) 293T cells were transfected with 4 μg of empty vector, or co-transfected with 2 μg of trTIPE3 plasmid and 2 μg of one of the following plasmids: empty vector, PI3KE545, Myr (myristoylated) PIP5K, and HRasV12. Thirty-two hr after the transfection, cells were stained with trypan blue and the percentages of dead cells determined. (K) The sedimentation-based extraction assay was used to determine (i) the percentages of TopFluorPtdIns(4,5)P_2 extracted from 100 μM 20% TopFluorPtdIns(4,5)P_2+80% PC vesicles and the percentages of TopFluorPC extracted from 100 μM 20% TopFluorPC+80% PC vesicles, and transferred to supernatant by TIPE3 or trTIPE3 (left panel), and (ii) fractions of TIPE3 and trTIPE3 proteins not bound to the 20% TopFluorPtdIns(4,5)P_2+80% PC and 20% TopFluorPC+80% PC vesicles (right panel). Proteins were used at a concentration of 20 μM. Values represent means ± SD; * $p<0.05$, and ** $p<0.01$. *p*- indicates phosphorylated. The experiments were repeated at least two times with similar results.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Tumor xenograft study

Tumors were generated by subcutaneous injection of 2×10^5 cells in 100 □l PBS into the flanks of six-week-old nude mice. Mice were monitored every other day and tumor volume was calculated using the following equation: $\pi(\text{width}^2 \times \text{length})/6$. At day 10 after the cell injection, mice were euthanized. Two hours before euthanasia, a group of mice were injected intraperitoneally with BrdU according to the manufacturer's instructions (Invitrogen). Tumors were excised, weighed, fixed, and paraffin-embedded for sectioning. Tumor sections were stained with hematoxylin and eosin, or with anti-BrdU or anti-cleaved caspase-3 according to the manufacturer's instructions (Cell Signaling Technology), and analyzed using ImageJ software. Student's *t*-test was used to evaluate the statistical significance of the differences in tumor growth, weight, BrdU incorporation, and caspase-3 cleavage.

Carcinogen-induced tumorigenesis study

Each seven-week-old female *Tipe3*$^{-/-}$ or wild type mouse was subcutaneously injected into the hind flank with 0.4 mg of 3-methylcholanthrene in 0.1 ml of corn oil. Mice were monitored weekly for the development of tumors and tumor volume was calculated as described above. At 17 weeks after the carcinogen injection, mice were euthanized and tumors were aseptically excised. Tumors were processed to establish fibrosarcoma cell cultures or fixed for histological analyses. For the establishment of *Tipe3*$^{-/-}$ and wild type fibrosarcoma cell cultures, tumor samples were finely minced and enzymatically digested by collagenase P (1.0 mg/ml, Roche) at 37°C for 15 min with rapid shaking. Then tumor samples were thoroughly washed and cultured in DMEM (Gibco) supplemented with 15% heat-inactivated fetal bovine serum (HyClone) and 100 U/ml penicillin and100 μg/ml streptomycin (Gibco) at 37°C, with 5% CO_2 for 2 weeks. Mann-Whitley *U* test was used to evaluate the statistical significance of the differences in tumor incidence and tumor growth between *Tipe3*$^{-/-}$ and wild type groups.

Human cancer studies

To study TIPE3 expression, we purchased two independently generated rabbit anti-TIPE3 antibodies - the first was against the RPNLKRICEGINKLLDEKVL peptide and the second against the MDSDSGEQSEGEPGC peptide of human TIPE3 (Wuhan Boster Biological Technology Inc., China). Both antibodies specifically recognize TIPE3, exhibit no cross-reactivity with other members of the TNFAIP8 family, and work for Western blot and immunohistochemistry (Sun et al., 2014). The first antibody was used for human lung and esophageal cancer studies whereas the second one was used for cervical and colon cancer studies. All tissue sections were 4 μm-thick and embedded in paraffin. They were first heated, dewaxed, and hydrated, followed by antigen retrieval in 10 mM citrate buffer solution, pH 6.0 (heated to boiling for 2-3 min). Endogenous peroxidase was blocked using 3% solution of hydrogen peroxide. The sections were then blocked with goat serum for 10 min, and incubated with rabbit anti-TIPE3 polyclonal antibodies at 4℃ overnight. Secondary staining was performed with HRP-conjugated anti-rabbit IgG using a MaxVision^{TM} Kit and a DAB Peroxidase Substrate kit (Maixin Co., Fuzhou, China). The sections were counterstained with hematoxylin. Normal rabbit IgG was used as a control for the primary antibody. All slides were independently analyzed by two pathologists in a blinded manner, and scored based on both intensity of TIPE3 expression and the percentage of TIPE3 positive cells as follows. The

intensity of TIPE3 expression: 0, no staining; 1, weak staining; 2, moderate staining; 3, strong staining. The percentage of TIPE3 positive cells: 0, <1%; 1, 1-20%; 2, 21-40%; 3, 41-60%; 4, 61%~80%; 5, ≥81%. The two scores for each slide were then combined to produce a final TIPE3 expression score. When there were discrepancies between the two pathologists, the average score was used. Mann-Whitney *U* test was used to evaluate the statistical significance of the results.

Cell culture and plasmids

Cell lines were obtained from the American Type Culture Collection (ATCC) and maintained in the suggested media. The following pharmacological inhibitors were used to treat the cells: LY29004 (50 μM, Cell Signaling Technology), PD98059 (100 μM, Promega), U-73122 (5 μM, Calbiochem), Bisindolylmaleimide I (4 μM, Cell Signaling Technology), and Rapamycin (100 nM, Cell Signaling Technology). Full-length TIPE3 cDNA was isolated from a C57BL/6 mouse brain by RT-PCR using TIPE3-specific primers, and subcloned into pRK5 and pBabe-hygro vectors with a Flag tag endcoded at the C- and/or N-terminus, as well as pEGFP-C3, pRK5-GST-HA PreScission [derived from pRK5-GST-HA PreScission p70 S6K1 (Addgene)], and pGEX-4T-1 vectors. TIPE3 and TIPE2 mutants were generated from TIPE3 and TIPE2 cDNAs, respectively, by PCR, and cloned as described for TIPE3. GRP1-PH-GFP was generated by cloning GRP1-PH fragment into pEGFP-C1 vector (Kavran et al., 1998). HRasV12 (pBabe HRasV12), PI3KE545 (pBabe HA PIK3CA E545K), myr-PIP5K (pWZL Neo Myr Flag PIP5K1B), PLCδ-PH-GFP (pEGFP-C1-PLCδ-PH), and AKT-PH-GFP (pEGFP-C1-AKT-PH) plasmids were purchased from Addgene. ShTIPE3 (GATCATGGTTGAGTGCTGTAACTTCA AGAGAGTTACAGCACTCAACCATGATC) and shScr (GATAGGTCTGGATGTATCAGTC TTCAAGAGAGACTGATACATCCAGACCTATC) were amplified together with U6 promoter by PCR and cloned into the pBabe-puro vector.

Plasmid DNA transfection and viral infection

293T cells were transfected with plasmid DNA using Fugene 6 (Promega) reagent according to the manufacturer's instructions. For virus production, pBabe (with puromycin or hygromycin resistance) with or without TIPE3, trTIPE3, HRasV12, shTIPE3, or shScr fragments and packaging constructs were co-transfected into 293T cells. After 24 and 48 hr, virus-containing medium was filtered and used to infect NIH3T3, NCI-H727, HT-29, or T24 cell lines in the presence of 6.5 mg/ml of polybrene (Millipore). Infected cells were selected using puromycin or hygromycin (Sigma) and used within 10 passages of infection. NIH3T3-HRasV12-vector (empty pBabe) and NIH3T3-HRasV12-TIPE3 cell lines were generated by (i) infecting NIH3T3 with HRasV12 containing virus, followed by selection using puromycin, and (ii) infection of the NIH3T3-HRasV12 cells with empty vector virus and TIPE3 containing virus, respectively, and then selected using hygromycin.

Real-time PCR

Total RNA was extracted from tissues or cells with TRIzol (Invitrogen) according to the manufacturer's instructions. Reverse transcription was performed using SuperScript II Reverse Transcriptase (Invitrogene) with oligo dT primers according to the manufacturer's instructions. Real-time PCR was carried out in an Applied Biosystems 7500 System with Power SYBR Green PCR Master Mix (Applied Biosystems). Murine *Tipe3* PCR fragments were amplified with 5'-CTC ATC CAG TGA GAA TCA AGC-3' and 5'-GGT TCA AAT CCG CAT CAG AGT-3'

primers. Human TIPE3, TIPE2, TIPE1, and TNFAIP8 PCR fragments were amplified with Hs_TNFAIP8L3_1_SG, Hs_TNFAIP8L2_1_SG, Hs_TNFAIP8L1_1_SG and Hs_TNFAIP8_3_SG QuantiTect Primers, respectively (Qiagen). Relative levels of murine and human TIPE family gene expression were determined using murine and human GAPDH, respectively, as the endogenous control. Levels of GFP mRNA in 293T cells transfected with GFP-expressing plasmids (pEGFP-C3 or pMigR1) were determined by quantitative real-time PCR using EGFP_1_SG QuantiTect Primers (Qiagen) and the corresponding plasmids as standards. All reactions were carried out in duplicates. Student's *t*-test was used to evaluate the statistical significance of the results.

Cell growth, cycle, and death assays

For the cell growth assay, cells were counted at denoted intervals on a hemocytometer slide using a light microscope. The growth of fibrosarcoma cells was assessed by staining them with crystal violet and determining absorbance at 550 nm. For the cell cycle assay, cells were stained with propidium iodide (Invitrogen) according to the manufacturer's instructions, and analyzed by flow cytometry for DNA content. For cell death assay, cells were stained with trypan blue and counted on a hemocytometer slide using a light microscope. For all assays, cells were cultured in duplicates. The Student's *t*-test was used to evaluate the statistical significance of the results.

Soft agar colony formation assay

Cells suspended in culture medium containing 0.3% of Noble Agar (Difco) were overlaid onto a bottom layer of solidified 0.6% Noble Agar in culture medium, at a concentration of 5×10^3 cells per well of six-well plates. Cells were cultured for 1-4 weeks until visible colonies were formed. Each cell line was assayed in duplicates. Colonies were analyzed with a light microscope, photographed, and quantified, and the colony sizes were analyzed using ImageJ software. More than thirty different fields were analyzed to assess the average number of colonies for each cell line and more than thirty colonies were analyzed to determine the average size of the colonies. Student's *t*-test was used to evaluate the statistical significance of the results.

GFP protein synthesis assay

293T cells were plated in 6-cm dishes (1×10^6/dish) and co-transfected with 1 μg of either pEGFP-C3 (5'cap-GFP) or pMigR1 (IRES-GFP) and 3 μg of one of the following constructs: pRK5, pRK5-TIPE3-Flag, pRK5 TIPE3 2Q-Flag, pRK5 TIPE3 3Q-Flag, pRK5 TIPE3 4Q-Flag, pRK5 TIPE3 60W-Flag, and pRK5-trTIPE3-Flag. Thirty-two hr later, the transfected cells were collected, and the intensity of cellular green fluorescence was analyzed by flow cytometry. The cells co-transfected with pRK5-trTIPE3-Flag were collected and analyzed 16 hr after the transfection to avoid trTIPE3-induced cell death. The results were evaluated using Student's *t*-test for statistical significance.

Immunofluorescence staining

For staining PtdIns(4,5)P_2 on plasma membrane with specific antibodies, NIH3T3 cells were transfected with pEGFP-C3, pEGFP-TIPE3, and pEGFP-trTIPE3 using PolyFect Transfection Reagent (Qiagen) according to the manufacturer's instructions. Thirty-two hours later, cells were stained as described by Hammond et al. and Sharma et al. with minor modifications (Hammond et al., 2009; Sharma et al., 2008). Briefly, cells were fixed with 4%

formaldehyde and 0.2% glutaraldehyde in HBS (25 mM HEPES, 150 mM NaCl, pH 7.4) buffer for 1 h at 4°C, permeabilized and blocked with 0.5% saponin in HBS buffer containing 3% BSA (fatty acids free, Sigma) for 2 h at 4°C, and then stained with primary anti-PtdIns(4,5)P_2 (1:100) (Echelon) in HBS/3% BSA/0.5% saponin overnight at 4°C and secondary anti-mouse IgM (Fab fragment) labeled with Alexa Fluor 555 (1:2000) (Invitrogen) in HBS/3% BSA/0.5% saponin for 2 h at 4°C. For staining PtdIns(4,5)P_2 in nucleus with specific antibodies, NIH3T3 cells were transfected with pEGFP-C3, pEGFP-TIPE3, or pRK5-TIPE3-Flag using PolyFect Transfection Reagent according to the manufacturer's instructions. Thirty-two hours later, cells were fixed with 3% formaldehyde in HBS buffer for 15 min at 37°C, permeabilized with 0.1 % Triton X-100 in HBS/3% BSA buffer for 10 min at room temperature (RT), blocked with 3% BSA in HBS for 50 min at RT and then, stained with primary anti-PtdIns(4,5)P_2 (1:100) in HBS/3% BSA for 1 h at RT, secondary anti-mouse IgM (Fab fragment) labeled with Alexa Fluor 555 (1:2000) and anti-Flag antibody labeled with Alexa Fluor 488 (1:300) (Cell Signaling Technology) in HBS/3% BSA for 1 h at RT. For staining phosphoinositides with specific PH-domains, NIH3T3 cells were co-transfected with pRK5, pRK5-TIPE3-Flag, or pRK5-trTIPE3-Flag and one of the following plasmids: PLCδ-PH-GFP, AKT-PH-GFP, and GRP1-PH-GFP using PolyFect Transfection Reagent. Thirty-two hr later, cells were fixed with 3% formaldehyde in HBS buffer for 15 min at 37°C, permeabilized with 0.1 % Triton X-100 in HBS/3% BSA for 10 min at RT, blocked with 3% BSA in HBS for 50 min at RT and then, stained with primary anti-Flag antibody (1:2000) in HBS/3% BSA for 1 h at RT and secondary anti-mouse IgG antibody (Fab fragment) labeled with Alexa Fluor 555 (1:3000) (Invitrogen) in HBS/3% BSA for 1 h at RT. In all experiments, immunofluorescence staining with fluorescently labeled secondary antibody only was used as controls. All slides were dried and covered with ProLong Gold with DAPI (Invitrogen). Images were acquired on a Zeiss LSM 510 NLO/META confocal microscope and analyzed using LSM Image Browser (Zeiss) and NIS-Elements BR3.10 (Nikon) software. All images shown are representatives of at least two independent experiments. More than thirty fields of cells expressing either TIPE3-GFP, GFP, or TIPE3-Flag were analyzed in each set of PtdIns(4,5)P_2 immunofluorescence experiments. Each field contained at least one cell expressing TIPE3-GFP, GFP, or TIPE3-Flag, and two non-transfected cells. Chi-squire test was used to evaluate the statistical significance of the results. The sub-cellular distribution of fluorescence signals was determined for at least seven individual cells per experiment using line intensity profiles (Stauffer et al., 1998). Student's *t*-test was used to evaluate the statistical significance of the results.

Western blot

For Western blot estimation of proteins, cells were lysed for 20 min at 4°C in 1% Triton X-100 lysis buffer containing protease and phosphatase inhibitors. Cell debris was removed by centrifugation at 14,000 × g for 20 min at 4°C. In subcellular fractionation experiment membrane and cytoplasmic proteins were separated using Qproteome Cell Compartment Kit (QIAGEN) according to the manufacturer's instructions. The protein concentration of the lysates was determined by BCA assay (Pierce). Equal amounts of total protein were resolved by SDS-PAGE, transferred to membranes, immunoblotted with specific primary and secondary antibodies, and the signals were detected by chemiluminescence (Pierce). Primary antibodies for p-AKT(T308), AKT, p-ERK1/2 (T202/Y204 of ERK1 and T185/Y187 of ERK2), ERK1/2, p-p70 S6K (T389), cyclin D1, and cleaved caspase-3 were purchased from Cell Signaling Technology and used

according to the manufacturer's instructions. Anti-HRas (used at dilution 1:500) and anti-α1 sodium potassium ATPase antibodies (1:1000) were from Abcam, and anti-Flag (1:2000), anti-β-actin (1:3000) and anti-GAPDH antibodies (1:3000) were from Sigma. Anti-TIPE3 antibodies raised against the TIPE3 TH-domain were used at a concentration of 0.15 μg/ml. These antibodies specifically recognize TIPE3 protein and exhibit no cross-reactivity with other members of the TNFAIP8 family. HRP-conjugated secondary anti-mouse and anti-rabbit IgG (1:1500) were purchased from GE Healthcare. The densitometric quantification of Western blot signals was performed using ImageJ software. Paired *t*-test was used to evaluate the statistical significance of the results.

TIPE3 purification for crystallization

Different constructs of human TIPE3 were screened for expression and behavior in both bacterial and insect cells. For selected bacterial expression system, TIPE3 sequence was codon-optimized and cloned into a modified pGEX-4T2 (GE Healthcare) vector with the drICE cleavage site. The *Escherichia coli* BL21(DE3) cells transformed with pGEX-4T2 TIPE3 vector were incubated at 37°C for 3 hr, followed by TIPE3 induction with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at OD_{600} of ~1.0. After growing for 14 hr at 15°C, the cells were harvested and re-suspended in TBS (25 mM Tris-HCl, 1 M NaCl, pH 8.0) buffer. The cells were lysed by sonication and cell debris was removed by centrifugation at 13000 rpm for 1 hr at 4°C. After centrifugation, the supernatant was loaded onto Glutathione Sepharose 4B resin (GE Healthcare) and washed with TBS buffer. The fusion protein was digested on column by drICE protease at 4°C overnight and eluted with TBS buffer. The eluate was concentrated to about 15 mg/ml and incubated with trypsin (final concentration of 35 μg/ml) for 30 min at RT. The protein was then applied to Superdex-200 (GE Healthcare) in TBS buffer, and the peak fraction was collected for crystallization with a final concentration of 10 mg/ml.

Estimation of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 cellular levels

Cellular levels of PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 were estimated using protein-lipid overlay assay with GST-PLCδ-PH and GST-GRP1-PH domains and dot blot with anti-PtdIns(4,5)P_2 or anti-PtdIns(3,4,5)P_3 antibody. For protein-lipid overlay assay, phosphoinositides were isolated from the cells as described (Clark et al., 2011) and spotted on membranes. The membranes were blocked with 3% BSA in PBS-0.1% Tween-20 (PBS-T) for 1 h at RT and incubated with GST-PLCδ-PH or GST-GRP1-PH (1.0 μg/ml, Echelon) in PBS-T with 3% BSA for 1 h at RT. Proteins were detected with anti-GST-HRP antibody (Cell Signaling Technology). In control protein-lipid overlay assays membranes were stained with anti-GST-HRP only. For normalization, part of the cell cultures were lysed in 1% SDS lysis buffer containing protease and phosphatase inhibitors, sonicated, spotted on membranes and immunoblotted with murine anti-β-actin (1:4000) and HRP-conjugated secondary anti-mouse IgG (1:3000). For dot blot studies, cells were lysed in 1% SDS lysis buffer containing protease and phosphatase inhibitors, and sonicated (Barrero-Villar et al., 2008). Lysates were normalized as described above. The normalized lysates were then spotted on membranes, immunoblotted with primary anti-PtdIns(4,5)P_2 (1:1000, Echelon) or anti-PtdIns(3,4,5)P_3 (1:200, Echelon) and the secondary HRP-conjugated anti-mouse IgM (1:2000, Invitrogen) or Flag-HRP antibody (1:1000, Sigma). Signals were detected by chemiluminescence (Pierce) and quantified by desitometry using ImageJ software. Paired *t*-test was used to assess the statistical significance of the results.

Screening for lipid binding using protein-lipid overlay assay

The membrane lipid strips containing triglyceride, PtdIns, PtdIns(4)*P*, PtdIns(4,5)P_2, PtdIns(3,4,5)P_3, phosphatidylserine (PS), phosphatidylethanolamine (PE), phosphatidic acid (PA), diacylglycerol (DAG), cholesterol, phosphatidylcholine (PC), sphingomyelin, phosphatidylglycerol (PG), 3-sulfogalactosylceramide, and cardiolipin, and PIP strips containing lysophosphatidic acid (LPA), lysophosphocholine (LPC), PtdIns(3)*P*, PtdIns(4)*P*, PtdIns(5)*P*, PtdIns(3,4)P_2, PtdIns(4,5)P_2, PtdIns(3,5)P_2, PtdIns(3,4,5)P_3, PE, PC, sphingosine 1-Phosphate (S1P), PA, PS were purchased from Echelon. The strips were blocked with 3% BSA in PBS-T for 1 h at RT. Recombinant TIPE3 and TIPE3 mutants with a Flag tag at the N-terminus were purified from 293T cells using Dynabeads Protein G (Invitrogen) coated with Flag antibodies (Sigma), and eluted from the beads with Flag-peptides (Sigma). Eluate samples for the control group were obtained by subjecting the lysates of 293T-pRK5 cells to the same purification process described above. Recombinant GST and GST-TIPE3-Flag proteins were affinity-purified from *Escherichia coli* BL21(DE3) cells (Stratagene) using Glutathione MagBeads (GenScript). GST was eluted from the beads with reduced glutathione; TIPE3-Flag was eluted by proteolytic cleavage of GST-TIPE3-Flag on the beads with thrombin (Amersham). Eluates were then concentrated, and depleted of Flag-peptides, reduced glutathione, or other contaminants by dialysis with Amicon Centrifugal Filters (Millipore). GST-PLCδ-PH was purchased from Echelon. The strips were incubated with 10 nM of purified recombinant proteins in PBS-T with 3% BSA for 1 h at RT. For Ins(1,4,5)P_3 competition experiments, 10 nM of recombinant proteins were pre-incubated with 15 μM Ins(1,4,5)P_3 (Echelon) for 30 min at RT before being used for PIP strip staining. Bound proteins were detected by immunoblot with anti-Flag-HRP or anti-GST-HRP (Millipore) using chemiluminescence reagents. Control protein-lipid overlay assays were performed following the same procedure using the control eluate instead of Flag-tagged proteins.

Phosphoinositide binding, extraction, and transfer assays

Dioleoylphosphatidylcholine (DOPC), brominated distearoyl PC (brominated PC), and TopFluor PtdIns(4,5)P_2 were purchased from Avanti Polar Lipids. PtdIns(4)*P*, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3 were purchased from Cell signals. Small unilamellar vesicles (SUVs) were produced as described (Lee and Lemmon, 2001). Recombinant GST-TIPE3, GST-TIPE3 4Q, and GST-trTIPE3 were purified from 293T cells using Glutathione MagBeads. TIPE3 and TIPE3 4Q were isolated by cleavage of GST-TIPE3 and GST-TIPE3 4Q proteins, respectively, with PreScission Protease (GE Healthcare), and dialyzed using Amicon Centrifugal Filters. Purified PLCδ-PH domain was kindly provided by Dr. Mendrola (University of Pennsylvania). Purified trypsin inhibitor of *Glycine max* was purchased from Sigma. Sedimentation-based binding assays were performed as described (Lee and Lemmon, 2001) with minor modifications: i.e., 20 μM of TIPE3, TIPE3 4Q, or Trypsin inhibitor and 10% PtdIns(4,5)P_2 [10% PtdIns(4,5)P_2 + 10% DOPC + 80% brominated PC], 10% PtdIns(4)*P* [10% PtdIns(4)*P* + 10% DOPC + 80% brominated PC], 10% PtdIns(3,4,5)P_3 [10% PtdIns(3,4,5)P_3 + 10% DOPC + 80% brominated PC] or 100% PC (20% DOPC + 80% brominated PC) SUVs were used in the assays, and the relative amounts of proteins in supernatants and pellets were determined by silver staining of SDS-PAGE gels containing the resolved proteins. In sedimentation-based PtdIns(4,5)P_2 extraction assays, 100 μM SUVs composed of 20% TopFluorPtdIns(4,5)P_2 and 80% DOPC were mixed with purified TIPE3 (5 – 40 μM), trTIPE3 (20 μM) or PLCδ-PH (5 – 40 μM), incubated 1 h at RT, and

centrifuged as described (Lee and Lemmon, 2001). In sedimentation-based PC extraction assays, 100 μM SUVs composed of 20% TopFluorPC and 80% DOPC were mixed with purified TIPE3 (20 μM) and trTIPE3 (20 μM), incubated 1 h at RT, and centrifuged as described (Lee and Lemmon, 2001). The fluorescence intensity of supernatants (corresponding to TopFluorPtdIns(4,5)P_2 or TopFluorPC extraction) was detected using a Synergy 2 fluorescence plate reader (BioTek). In sedimentation-based PtdIns(4,5)P_2 transfer assays, supernatants generated in TIPE3 sedimentation-based PtdIns(4,5)P_2 extraction assays were mixed with 250 μM 100% PC SUVs, incubated for 1 h at RT, and centrifuged as described (Lee and Lemmon, 2001). The pellets were solubilized in HBS buffer containing 0.25% Triton X-100. The fluorescence intensity of supernatants (corresponding to soluble TIPE3/TopFluorPtdIns(4,5)P_2) and solubilized pellet fractions (corresponding to TopFluorPtdIns(4,5)P_2 transfer into 100% PC SUVs) was detected as above. Student's *t*-test was used to assess the statistical significance of the results.

Surface plasmon resonance (SPR)-based assay

SPR-based assays were carried out using a BIAcore 3000 instrument measuring PtdIns(4,5)P_2 binding as described previously (Moravcevic et al., 2010; Sugiki et al., 2010). Briefly, 10% PtdIns(4,5)P_2 [10% PtdIns(4,5)P_2 + 90% DOPC], 3% PtdIns(4,5)P_2 [3% PtdIns(4,5)P_2 + 97% DOPC] or 100% PC (100% DOPC) SUVs were generated and immobilized on L1 sensor chip surfaces (BIAcore), resulting in signals of ~ 6500 to 8500 resonance units. GST-PLCδ-PH was kindly provided by Dr. Moravcevic (University of Pennsylvania). GST-PLCδ-PH (5 μM) binding to these surfaces was measured before and after injections of TIPE3 (40 μM) or TIPE3 4Q (40 μM) in HBS buffer pH 7.4 at a flow rate of 3 μl/min. SPR signals detected in sensorgrams were analyzed using BIAevaluation software (BIAcore). Signals for each experiment were corrected for background (DOPC) binding. The PtdIns(4,5)P_2 extraction by TIPE3 and TIPE3 4Q from 3% and 10% PtdIns(4,5)P_2-containing surfaces was quantified as reductions in GST-PLCδ-PH binding to these surfaces after TIPE3 or TIPE3 4Q had been flowed over the sensor chip. GST-PLCδ-PH binding to PtdIns(4,5)P_2-containing surfaces before TIPE3 or TIPE3 4Q exposure was set as 100% and GST-PLCδ-PH binding to PtdIns(4,5)P_2-containing surfaces after TIPE3 or TIPE3 4Q exposure was quantified as the percentage reduction. Student's *t*-test was used to assess the statistical significance of the results.

PI3K enzymatic assay

Active PI3K (p110α/p85α) was purchased from Echelon. SUVs were produced as described (Lee and Lemmon, 2001). PI3K enzymatic activity was measured using membrane capture assay as described (Knight et al., 2007) with minor modifications. Briefly, PI3K was assayed at a concentration of 2 μg/ml in HBS buffer with 10 mM $MgCl_2$, 50 μM ATP, 500 μg/ml BSA, 250 μM SUVs composed of 10% PtdIns(4,5)P_2 + 10% DOPC + 80% brominated PC, and in the absence or presence of increasing amounts of TIPE3 (0-200 nM). Before adding to the reactions, SUVs were pre-incubated with 0-200 nM of TIPE3 or BSA in HBS buffer for 1 h at RT. Reactions were initiated by adding ATP, and terminated by spotting on nitrocellulose membrane. Reactions of PI3K with 0-200 nM TIPE3 but without ATP and reactions with ATP and 0-200 nM TIPE3 but without PI3K were used as negative controls. The levels of PtdIns(3,4,5)P_3 in each reaction were measured by protein-lipid overlay assay with GST-GRP1-PH domain. Signals were detected by chemiluminescence (Pierce) and quantified by desitometry using ImageJ software. Paired *t*-test was used to assess the statistical significance of the results.

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