Regulation of TM4SF5-mediated tumorigenesis through induction of cell detachment and death by Tiarellic Acid

Suyong Choi[1,2], Sei-Ryang Oh[3], Sin-Ae Lee[1,4], Sung-Yul Lee[1,4], Kyungseop Ahn[3], Hyeong-Kyu Lee[3], and Jung Weon Lee[1,2,4,5]. [1]Cancer Research Institute, Cell Dynamics Research Center, Departments of [2]Tumor Biology, [4]Molecular & Clinical Oncology, College of Medicine, Seoul National University, Seoul 110-799, [3]Natural Medicine Research Center, Korea Research Institute of Bioscience and Biotechnology, Daejeon 305-806, Korea.

[5] To whom correspondence and proofs should be

Jung Weon Lee, Ph.D. Cancer Research Institute, College of Medicine, Departments of Tumor Biology, Molecular & Clinical Oncology, Seoul National University, 103, Daehangno, Jongno-gu, Seoul 110-799, Korea. (Phone) 82-2-3668-7030, (Fax) 82-2-766-4487, (E-mail) jwl@snu.ac.kr

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Summary

mRNA for transmembrane 4 L6 family member 5 (TM4SF5), a homolog of tumor antigen L6, was previously shown to be highly expressed in diverse tumors. We recently found that human hepatocarcinoma tissues also overexpressed TM4SF5 protein, in comparison to normal liver tissues. We also found that tiarellic acid (TA) caused cell detachment-related apoptosis in cells expressing endogenous or stably-overexpressing TM4SF5. When cells expressing TM4SF5 were treated with TA, we observed reduced phosphorylation of focal adhesion kinase, paxillin, and p130Cas, but not c-Src. TA treatment also caused focal adhesion loss and reduced cell adhesion, and increased the numbers of floating cells and apoptotic cells. These effects were blocked by overexpression of focal adhesion molecules, suggesting that treatment with TA mediates anoikis of TM4SF5-expressing cells. However, TM4SF5-null cells were not affected by TA, indicating that these effects occur specifically in TM4SF5-positive cells. TA administration reduced tumor formation in nude mice injected with TM4SF5-expressing cells, presumably through increased apoptosis in TM4SF5-positive tumors. These observations indicate that TM4SF5-positive tumorigenesis can be inhibited by TA via induction of cell detachment-related apoptosis, and suggest that TA may be developed as a putative therapeutic reagent against TM4SF5-positive tumorigenesis.

Introduction

Integrin-mediated cell adhesion triggers diverse intracellular signal transductions that regulate the activities and localizations of diverse signaling molecules, and consequently cell behaviors [1-3]. When integrins interact extracellularly to extracellular matrix (ECM) at focal adhesions, intracellular cytoplasmic tails of integrin subunits recruit diverse focal adhesion molecules including FAK, paxillin, p130Cas, c-Src, and *etc* [4]. Since the integrins at the focal adhesions are linked to actin filaments through protein complexes at their cytoplasmic tails [5, 6], inefficient assembly and activation of focal adhesion molecules and/or abnormal reorganization of actin filaments can cause cells to be round-shaped with concomitant loss of focal adhesions, leading to consequent detachment of cells from and disruption of the epithelial integrity on basement membrane [7].

However, cancer cells due to multiple mutations and genomic instabilities may be allowed to disseminate from a primary tumor by abnormal alteration of cell-ECM interactions and cell-cell contacts [8]. In the steps of disruption of basement membrane and migration/invasion through stromal region for cancer metastasis, integrin-mediated adhesion of cancer cells to ECM may critically affect the metastatic potential of cells [9-11]. Among disseminated cancer cells, only cells survived anoikis may travel to distant sites via blood and lymphoid vessels, and eventually attach to a site and proliferate as metastatic tumors [12].

Therefore, it would be useful to find reagent(s) to cause apoptosis or anoikis of tumorigenic cells without any effects on normal cells, when anti-tumorigenic or metastatic reagents are being screened.

TM4SF5 (also known as L6H) is a homologue of tumor-associated antigen L6 (TM4SF1) and forms a four-transmembrane L6 superfamily with L6, IL-TMP, and L6D [13]. TM4SF5 is highly expressed in diverse tumor types [14, 15]. Tetraspanins (also known as TM4SF or tetraspans) are a group of hydrophobic proteins of 25 ~ 50 kDa with four transmembrane domains, two extracellular loops, and two short cytoplasmic tails [16]. The extracellular loop 2 (EC2) of genuine tetraspanins has a variable region that mediates specific protein-protein interactions and a conserved region that mediates homodimerization [16]. The EC2 loop of TM4SF5, a member of a four-transmembrane L6 superfamily, however, is relatively divergent and has 2 cysteine residues, whereas the EC2 of genuine tetraspanins has a variable region and conserved 4 cysteines for disulfide bonding while correct folding [13]. Four transmembrane domains of tetraspanins are suggested to be responsible for intra- and inter-molecular interactions during biosynthesis and organizing the 'tetraspanin-web' on cell surface [16]. Tetraspanins form complexes with integrins to collaboratively perform their roles in cell adhesion, proliferation, and motility [17, 18]. It was previously shown that KAI1/CD82 expression in Du145 prostate cells down-regulated p130Cas leading to

suppression of cell motility, and overexpression of p130Cas in the cells reversed the inhibited motility [19]. Meanwhile, we recently found that TM4SF5 overexpression in hepatocytes caused actin reorganization through regulation of FAK and RhoA activity, leading to losses of cell-cell adhesion and contact inhibition [20]. We have also observed that ectopic TM4SF5 in fibroblasts was shown to interact with integrin α2 to regulate focal adhesion turnover and actin reorganization [21]. Therefore, it is likely that TM4SF5 may cross-talk with the integrins to regulate cellular functions.

While trying to identify reagents to regulate TM4SF5-mediated cell proliferation, we found that tiarellic acid [TA, [22]] could cause cell detachment-related death in a TM4SF5 expression-dependent manner. TA treatment decreased phosphorylation of focal adhesion molecule and cell adhesion, enhanced apoptosis of TM4SF5-expressing (but not -null) cells, and interfered with TM4SF5-mediated tumor formation in nude mice. Altogether, these observations suggest that TA may be a putative anti-tumorigenic reagent specifically against TM4SF5-positive tumors.

Materials and Methods

Cells: Pooled stable clones (control SNU449Cp and TM4SF5-expressing SNU449Tp cell clones) and single cell-driven stable clones (TM4SF5-expressing SNU449T16) were prepared by infecting SNU449 hepatocytes (Korean Cell Bank, Korea) with retrovirus for empty pLNCX or pLNCX with TM4SF5 [20]. Stable and TM4SF5-null parental cells were maintained in RPMI-1640/10% FBS/0.25 μg/ml gentamycin (Calbiochem, San Diego, CA) with or without 200 μg/ml G418 (A.G. Scientific Inc., San Diego, CA), respectively, at 37^{o}C and 5% CO_2. Other cell lines with or without endogenous TM4SF5 (Korean Cell Bank) were maintained in either DMEM-H or RPMI-1640 media with 10% FBS (JBI Inc., Daegu, Korea).

Preparation of Tiarellic acid **(TA)**: Whole plants of *Tiarella polyphylla* collected from Ullung island (Kyungsang-Bukdo, Korea) were extracted with MeOH, concentrated, suspended in water, partitioned successfully with EtOAc prior to column chromatography on silica gel eluting with hexane followed by hexane-EtOAc mixtures of increasing polarity and finally with $CHCl_3$–MeOH (1:1) mixture, as previously explained [23]. HPLC (MeOH 58% + 0.2% TFA 42%) of TA (0.1 μg/μl, 3,23-dihydroxy-20(29)-lupen-27-oic acid, [22]) was performed using Finnigan Surveyor HPLC system with column of Perfectsil Target ODS-3.

Cell lysates preparation and Western blots: Whole cell lysates from cells under diverse

experimental conditions were prepared, as described previously [21]. In cases, cells were electroporated (at 950 μF and 250 V) with control GFP, wildtype FAK, paxillin, or p130Cas cDNA plasmid. One day after electroporation, cells were treated with TA (20 μM) for the indicated periods. Cells cultured normally or treated with TA at different concentrations for the indicated periods were harvested using RIPA buffer [21]. Control or tumor tissues from nude mice were frozen immediately after surgery using liquid N_2. Tissues was homogenized in the liquid N_2 and extracted using 0.1% SDS containing RIPA buffer, prior to spin (13000 x *g*, 30 min) at 4°C. Standard Western blots of lysates were performed by using antibody against phospho-Y^{397}, Y^{407}, Y^{577}, Y^{861}, and Y^{925}FAK (Invitrogen, Carlsbad, CA), Bcl-2, Bax, phospho-Y^{416}Src, c-Src, $p15^{INK4B}$, $p16^{INK4A}$ (Santa Cruz Biotech., Santa Cruz, CA), FAK, p130Cas, paxillin, phospho-Y^{118}paxillin, active caspase-3 (BD Bioscience, San Jose, CA), α-tubulin (Sigma, St Louis, MO), phospho-S^{473}, phospho-T^{308}Akt, Akt (Cell Signaling Tech., Danvers, MA), or TM4SF5 (homemade).

Anti-TM4SF5: Rabbit polyclonal antibody was produced against amino acid residues from 120 to 132 of TM4SF5 (NM-003963) conjugated with keyhole limpet hemocyanin (KLH, A & Pep Inc., Yeongi-Gun, Korea). The sera were further purified by affinity chromatography using protein A/G-sepharose (Invitrogen).

Immunofluorescence microscopy: Cells were treated with DMSO or 20 μM TA for 24 h

before being replated on fibronectin (10 μg/ml) -precoated coverslips for an additional 2 h incubation in the presence of DMSO or 20 μM TA. Fluorescent staining was performed, as described previously, for analysis by fluorescent microscopy (BX51, Olympus, Japan) [21].

Mouse and tumor xenografts: Four- or five-week-old female nude (BALB/cAnNCrjBgi-nu, Orient Co. Ltd., Seongnam, Korea) mice were housed and handled, according to the procedures in the Seoul National University Laboratory Animal Maintenance Manual and IRB approval. Mice were injected subcutaneously in a flank with 5 x 10^6 of viable SNU449Cp, SNU449T16, or CT26 murine colon carcinoma cells. Tumor volumes were measured with a caliper and calculated using the formula: Volume = (**a** x $\mathbf{b}^2$)/2, where **a** was the width at the widest point of the tumor and **b** was the maximal width perpendicular to **a**. Intraperitoneal (IP) injection of TA (at 3 or 30 mg/kg body weight in 5% DMSO) to mice with TM4SF5-mediated tumors (~ 200 mm^3 of calculated tumor volume) was performed every other day for the indicated periods.

***Human liver tissue sample*:** Normal and tumor liver tissues were obtained from patients in the Kyungpook National University Hospital (Daegu, Korea) from March to April, 2007 with informed consent and IRB approval. Clinical and biological information was available for the tissue samples.

Tumor histology: Tumor tissues were removed about 6 weeks after injecting SNU449T16

cells or after TA IP injection for one month, fixed with formalin, and embedded in paraffin. Serial sections (6 μm thick) cut from each paraffin block were stained with anti-active caspase-3, prior to visualization by fluorescent microscopy (BX51, Olympus, Japan).

Viability assay: Cells were seeded into 24 well culture plates at 1 x 10^5 cells/well. One day later, cells in triplicate were treated with TA at different concentrations for the indicated periods. After incubation, viable cells after trypan blue staining or total floating cells were counted.

Immunoprecipitation: Cell lysates were immunoprecipitated with p130Cas, integrin α5, or FAK antibody. The p130Cas immunoprecipitates were immunoblotted with anti-phospho-Tyr, FAK, or paxillin, whereas the FAK or integrin α5 immunoprecipitates were immunoblotted for integrin α5 or pY^{397}FAK, respectively, as explained previously [24].

Cell adhesion assay: Cells treated with DMSO or 20 μM TA for 24 h were trypsinized and suspended in RPMI-1640 containing 1% BSA. The cells were then processed for assay of adhesion on fibronectin for 1 h, as explained previously [25].

DNA content assay: The DNA content analysis via propidium iodide staining was performed for cells with or without TA treatment, or for cells transfected with GFP, FAK, paxillin, or p130Cas wildtype with or without TA treatment (20 μM for 24 h), as previously explained [26].

Statistical analysis: The Student's *t*-test was performed for comparison of mean values to see if the difference is significant. p values ≤ 0.05 were considered significant.

Results

TA treatment results cell-detachment related death of TM4SF5-expressing cells

While investigating to see whether TM4SF5 might be associated with tumorigenesis, we found that TM4SF5 was significantly overexpressed in liver tumor tissue obtained from hepatocarcinoma patients, compared to normal liver tissue (Figure 1A). To identify reagents that have an effect on cells expressing TM4SF5, we screened diverse natural product-driven chemicals to observe selective cytotoxic effects against TM4SF5-expressing cells. We used TM4SF5-negative parental SNU449 and control stable SNU449Cp hepatocytes and TM4SF5-positive SNU449Tp and SNU449T16 stable cells (Figure 1B, upper) to examine cytotoxic sensitivity to a variety of lupane-derived triterpenoid plant compounds. Among the compounds we tested, tiarellic acid (TA, Figure 1C) was found to inhibit growth of TM4SF5-expressing cells (Figure 1D); although TA treatment did not change TM4SF5 expression levels (Figure 1B, lower). TM4SF5-expressing SNU449Tp cells treated with TA showed cell death accompanied by rounded cell morphology (data not shown), whereas TM4SF5-null SNU449Cp cells were not affected by TA treatment. Treatment with 20 μM TA even for one day decreased the number of TM4SF5-expressing cells (Figure 1D). These observations indicate that TA might target other molecules(s) than TM4SF5 itself in the initiation of cell death.

When we analyzed trypan blue stain-positive and floating cells after DMSO or TA treatment, we found that TA treatment increased the number of floating SNU449Tp cells, but not of control SNU449Cp cells (Figure 1E). TA treatment of SNU449T16 cells also resulted in Akt inactivation, Bcl-2 reduction, Bax induction, and caspase-3 activation, whereas no effects were seen in control SNU449Cp cells (Figure 1F). In addition, analysis of cellular DNA content after treatment with 20 μM TA for 24 h resulted in significant sub-G1 populations of SNU449Tp and T16, but not of control SNU449Cp cells (Figure 1G). The TA-mediated apoptosis rate was less than the overall loss in viability (Figure 1D), presumably due to different sensitivities of experimental methods and/or technical difficulties to collect all apoptotic bodies during sample preparation for FACS analysis. Therefore, these observations suggest that TA causes cell detachment-related death of TM4SF5-expressing cells.

We next evaluated whether the TA treatment of cells overexpressing TM4SF5 was valid for cells that endogenously express TM4SF5. We examined effects of TA treatment at 5 or 20 μM on cell growth and survival using diverse cell lines with or without endogenous TM4SF5 (Figure 2A). Growth of TM4SF5-null cells (including Hek293 and COS7 fibroblast, MDA-MB453 breast, SNU638 and SNU668 stomach, and SNU398 liver epithelial cells) were not affected by TA treatment; although Hek293 cells showed a slight retard of

growth rate after TA treatment, they still increased in cell number (Figures 2B left and data not shown). However, cells with endogenous TM4SF5 expression (including SNU368, HepG2, and Huh7 hepatocytes) showed growth suppression after TA treatment, leading to the eventual decrease in cell number (i.e., cell death) over time (Figure 2B, right).

TA-mediated inhibition of focal adhesion molecule activation and focal adhesion formation

To examine the molecular basis for TA-mediated cell detachment and death, we analyzed signaling molecules known to participate in focal adhesion upon integrin-mediated cell adhesion. SNU449 stable cell lines were treated with TA in a dose- and time-dependent manner. SNU449Cp or SNU449T16 cells were treated with varying TA concentrations for 24 h prior to immunoblot analysis. SNU449T16 cells treated with 20 μM TA showed reduced FAK and paxillin phosphorylation and p130Cas protein levels, whereas SNU449Cp cells were not affected (Figure 3A). TA treatment decreased FAK and paxillin phosphorylation and p130Cas protein levels in a time-dependent manner in SNU449T16, but not in SNU440Cp cells (Figure 3B). The TM4SF5-dependent inhibitory effects of TA on focal adhesion molecule phosphorylation and activity were observed in both SNU449Tp and T16 cell lines (Figure 3C). In addition, TA treatment decreased p130Cas phosphorylation and the physical association among FAK, p130Cas, and paxillin (Figure 3D). These

observations indicate that TA treatment affected phosphorylation and complex formation of focal adhesion molecules only in TM4SF5-expressing cells. In addition, TA-mediated effects on focal adhesion molecule phosphorylation were examined in cells that express endogenous TM4SF5 and in null cell lines. The inhibitory effects of TA treatment were observed also in cells endogenously expressing, but not lacking, TM4SF5 (Figure 3E). Since TA suppressed focal adhesion molecule phosphorylation and complex formation, focal adhesion formation would be likely be affected by TA treatment. We thus analyzed focal adhesion formation in TA-treated and -untreated cells by performing immunostaining for Tyr397-phosphorylated FAK (pY^{397}FAK) or Tyr118-phosphorylated paxillin (pY^{118}paxillin). Although TA treatment did not affect focal adhesion formation in TM4SF5-null cells, focal adhesions in TM4SF5-expressing cells were lost after TA treatment (Figure 4A). TA-mediated loss of focal adhesions appeared to involve a dissociation between integrin α5 and pY^{397}FAK in TM4SF5-expressing cells (Figure 4B), being consistent with integrin-mediated recruitment of focal adhesion molecules such as pY^{397}FAK [21]. Furthermore, since focal adhesions are linked to each other via stress fibers [27], we also stained actin in TA-treated and -untreated cells. Cells expressing TM4SF5 showed abnormal actin organization with insignificant stress fibers after TA treatment, whereas TM4SF5-null cells did not (Figure 4C). The loss of focal adhesions and stress fiber formation in TA-treated, TM4SF5-expressing cells

could contribute to the observed cell rounding after TA treatment (data not shown).

Since TA treatment caused inactivation and disassembly of focal adhesion molecules, possibly contributing to a rounded morphology, and resulting in detachment-related apoptosis of TM4SF5-expressing cells, we next wondered whether TA treatment affected cellular adhesion. Cells treated with 20 μM TA for 24 h were collected and reseeded on fibronectin-precoated dishes for 1 h before determination of the degree of adhesion. Adhesion of TM4SF5-null SNU449 and SNU449Cp cells were not affected by TA treatment, whereas TM4SF5-expressing SNU449Tp and T16 cells pretreated with TA showed significantly reduced adhesions, compared to the control vehicle-treated cells (Figure 4D). This observation appeared to be consistent with the observed TA-mediated detachment and death of TM4SF5-positive cells in Figure 1.

Overexpression of focal adhesion molecules blocked the TA-mediated effects

We next evaluated whether overexpression of FAK, paxillin, or p130Cas would block the effects of TA treatment, assuming that their expression to higher levels would result in more focal adhesion formation and activation. To explore this, we introduced each cDNA into SNU449T16 cells via electroporation. Due to transient overexpression of FAK, paxillin, or p130Cas, TA treatment was performed I day after electroporation. TA treatment of SNU449T16 cells introduced with control plasmid (for GFP) showed time-dependent

inactivation of focal adhesion molecules and activation of caspase-3, supporting our hypothesis of TA-mediated apoptosis. However, SNU449T16 cells introduced with wildtype FAK, paxillin, or p130Cas resulted in certain degrees of blocking TA-mediated effects (Figure 5A). Furthermore, overexpression of the focal adhesion molecules also blocked TA-mediated increases in the sub-G1 population (Figure 5B). Therefore, the TA-mediated effects on TM4SF5-expressing cells could be overcome to a certain degree by overexpression of focal adhesion molecules.

TA interfered with TM4SF5-mediated tumor formation in nude mice

We next evaluated whether TM4SF5 mediated tumor formation in nude mice and whether TA administration might interfere with tumorigenesis. Nude mice injected with TM4SF5-expressing SNU449T16 cells formed significant tumors, whereas injection with TM4SF5-null SNU449Cp cells did not form tumors (Figure 6A). When TA was given intraperitoneally every other day for 30 days to mice with established tumors (~ 200 mm^3 of calculated tumor volume), TM4SF5-mediated tumor formation was decreased. Administration of TA at 3 or 30 mg/kg body weight decreased the tumor volumes to 46.0% or 22.1%, respectively, while body weight increased normally, indicating no significant toxic side effects (Figure 6A). Although we did not determine whether TA caused any local side effects in the TA-treated mice such as blood pressure decrease and mucosal damage, an intraperitoneal administration

of TA at 2 g/kg did not cause any abnormal behaviors or abnormalities in organ structures until sacrifice after 2 weeks (S-R Oh and H-K Lee, unpublished observation). Western blot analysis using control tissue around the spot injected with control SNU449Cp cells and tumor tissue of SNU449Tp-injected mice revealed that TM4SF5-mediated tumor tissue showed Akt activation and caspase-3 inactivation, indicating signaling activities for tumor formation (Figure 6B, lanes 1 to 2). However, TA administration decreased Akt activation, increased $p15^{INK4B}$ or $p16^{INK4A}$ cyclin-dependent kinase inhibitor (CKI) levels, and enhanced caspase-3 activation, indicating that TA caused growth inhibition and apoptosis in the TM4SF5-mediated tumors (Figure 6B, lanes 2 to 4). Immunohistochemical staining for active caspase-3 was positive in TM4SF5-mediated tumor tissue obtained from mice that had been treated with TA, but not from mice treated with in vehicle DMSO (Figure 6C). Since we observed that TM4SF5-positive cells were undergoing apoptosis after TA treatment, it may be likely that doses higher than 30 mg/kg may cause cytostatic effects leading to dormancy of TM4SF5-mediated tumors in mice. On the other hand, nude mice injected with TM4SF5-negative CT26 murine colon carcinoma cells followed by TA treatment (30 mg/kg body weight) did not result in decreased tumor formation (Figure 6D). Therefore, TA specifically interfered with TM4SF5-mediated tumor formation in nude mice, presumably by enhancing apoptosis. These results suggest that TA might be developed further as a potential

therapeutic reagent for TM4SF5-positive tumors.

Discussion

This study provides evidence that TM4SF5-positive cell growth and tumor formation in nude mice can be negatively regulated by tiarellic acid (TA), a component purified from *Tiarella polyphylla* [22]. TA treatment resulted in the cell detachment-related death of TM4SF5-expressing cells, but not null cells; TA inhibited activation of and complex formation between focal adhesion molecules, blocked formation of focal adhesions, decreased adhesion property, and increased death in cells ectopically and endogenously expressing TM4SF5. TM4SF5 mRNA is overexpressed in diverse tumor types [14, 15] and we show in this study that TM4SF5 protein is overexpressed in human hepatocarcinoma tissue. Therefore, the significance of TA-mediated apoptosis in TM4SF5-expressing cells can be important for therapeutic purposes against TM4SF5-positive cancers, although the precise target of TA during the TA-mediated detachment-related death of TM4SF5-expressing cells is unknown.

Other genuine tetraspanins form tetraspanin-web structures by interacting with integrins to collaboratively perform their functions in cell adhesion, proliferation, and motility [17, 18, 28]. We have previously reported that TM4SF5 regulates focal adhesion turnover and actin reorganization in fibroblasts, depending on the association with integrin α2 [21]. In addition, TM4SF5 in hepatocytes was shown to modulate cellular morphology via regulation of FAK

and RhoA activities [20]. TM4SF5 may thus modulate architectural integrity and/or signaling activity of membrane proteins, including integrins and other membrane receptors. TA was previously shown to reduce MMP-1 expression and induce type 1-procollagen expression after ultraviolet irradiation of primary human skin fibroblasts [23], although it is not known whether the primary skin fibroblasts express TM4SF5 or not. Therefore, TA appears to regulate the behaviors of TM4SF5-expressing cells by influencing cell adhesion-related molecules. TA treatment of Hek293 cells lacking TM4SF5 resulted in a slight growth retard, although they still divided to positively increase cell numbers, whereas other TM4SF5-negative cell lines were not influenced (Figure 2B). Therefore, it cannot be ruled out that Hek293 cells may express a TM4SF5-related protein, such as L6 (TM4SF1), IL-TMP, and L6D, or that TA may also have alternative targets involved in Hek293 cell adhesion, which do not exist in SNU449 cells.

Lupane-derived triterpenoid compounds with a pentacyclic backbone like TA have been known for 20 years to have cytostatic activity [29]. Dehydrothyrsiferol, a triterpenoid, was recently shown to modulate integrin affinity without changing the expression of cell surface integrin expression, FAK, or Akt phosphorylation levels, leading to anoikis of human breast cancer cells [30]. TA is not soluble in water or culture media but in DMSO and would not generally affect cell adhesion property via functions as a detergent, since TA treatment caused

detachment-related death only of TM4SF5-expressing cells, but not -null cells. Our observations with chemical derivatives of TA suggest that a structural specificity of TA exists to inhibit proliferation and survival of TM4SF5-positive cells, indicating that a COOH group at C_{27} is important for the inhibitory effects on growth and survival of TM4SF5-positive cells (in preparation). On the other hand, tetraspanins or TM4SFs form massive complexes with integrins on the cell surface to function in cell adhesion [28]. Although attempts to coimmunoprecipitate TM4SF5 and integrin α5 in the absence of TA treatment of SNU449Tp or T16 cells failed, the connection between integrin and pY^{397}FAK was disrupted by TA treatment. Therefore, TA may interfere with the cross-talk between TM4SF5 and integrins, at the level of effectors downstream of the receptors, which may be important for regulation of focal adhesion assembly and cell-ECM adhesion.

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Figure legends

Figure 1. TA causes detachment-related death of ectopically TM4SF5-expressing cells. (A) Whole cell extracts from normal (N) or tumor (T) liver tissues from liver carcinoma patients were immunoblotted for TM4SF5 and α-tubulin. (B) Parental SNU449 and stable control SNU449Cp cells without or stable SNU449Tp and SNU449T16 cells with ectopic TM4SF5 expression [21] were confirmed for TM4SF5 expression. Whole cell extracts from subconfluent cells (upper) or cells treated with or without 20 μM TA for 24 h (lower) were prepared and used in immunoblots for the indicated molecules. (C) Chemical structure (left) and HPLC chromatogram of tiarellic acid (TA). (D and E) One day after cells were seeded into 24 well plate at 1 x 10^5 cells/well, cells were treated with DMSO or TA at different concentrations for 1, 2, or 3 days. After incubation, viable cells after staining with trypan blue were counted in triplicate using a hemocytometer (D). Alternatively, total floating cells at the indicated conditions were collected by spin-down and then counted (E). (F and G) Cells were seeded and 24 h later treated with 20 μM TA for the indicated periods (F) or 24 h (G). (F) Whole cell lysates were prepared and used in standard Western blots for the indicated molecules. (G) DNA content analysis after propidium iodide staining for 30 min was performed, as explained previously [26]. The percentage of the population with apoptotic sub-G1 DNA contents was included in each histogram. Data shown represent three independent experiments.

Figure 2. TA-mediated growth inhibition of endogenously TM4SF5-expressing cells. (A) Expression levels of endogenous TM4SF5 mRNA or protein in diverse cell lines were analyzed via RT-PCR and Western blot. Control GAPDH levels during RT-PCR were observed to be quite similar in comparisons between different cell lines. (B) To examine TA-mediated viabilities of diverse cell lines with or without endogenous TM4SF5, cells were separately seeded in triplicate (at an equal cell number) into 24 well culture plates. One day later, DMSO or TA at different concentrations were treated to cells for the indicated periods, before counting of viable cells, as in Figure 1D. Data shown represent three independent experiments.

Figure 3. TA causes inactivation of and complex disassembly among focal adhesion molecules in a dose- and time-dependent manner. (A to E) Cells were seeded to 60 mm culture dishes at about 40% density. One day after, cells were treated with DMSO or TA at diverse concentrations for the indicated periods. After incubation, lysates were prepared and used in standard Western blots for the indicated molecules. (D) An equal amount of proteins for each condition was immunoprecipitated with anti-p130Cas, before immunoblots with anti-phospho-Tyr to reveal p130Cas phosphorylation, or anti-FAK or -paxillin to determine

complex formation among the molecules. Data shown represent three independent experiments.

Figure 4. TA causes detachment of TM4SF5-expressing, but not -null, cells via loss of focal adhesion and stress fiber. (A to C) Cells were treated with DMSO or 20 μM TA for 24 h, before being replated on fibronectin (10 μg/ml) -precoated coverslips. Replated cells were then incubated for 2 h in the presence of DMSO or 20 μM TA, before immunofluorescent staining for pY^{397}FAK or pY^{118}paxillin (A), or actin staining using phalloidin-conjugated Rhodamine (C). (B) Lysates were prepared from subconfluent SNU449T16 cells for immunoprecipitation with anti-FAK or -integrin α5 (P1D6 clone). Immunoprecipitates or lysates were immunoblotted for the indicated molecules. (D) Parental (P) and stable SNU449 cell clones were reseeded, and one day later treated with DMSO or TA (20 μM) for 24 h. After incubation, cells were detached and resuspended into RPMI1640 with 1% BSA. An equal number of cells for each condition were evaluated for adhesion (for 1 h) onto fibronectin (10 μg/ml), as explained in Materials and Methods. Relative adhesions compared to basal adhesion of the parental SNU449 (P) cells (at 100%) were calculated from measurements in triplicate, and mean ± standard deviation values were graphed. * and ** indicate statistical insignificance ($p > 0.05$) and significance ($p \leq 0.05$),

respectively. Data shown represent three independent experiments.

Figure 5. Overexpression of focal adhesion molecules blocked the TA-mediated effects. (A and B) SNU449T16 cells were electroporated with cDNA plasmid for GFP, FAK, paxillin, or p130Cas wildtype, as explained in Materials and Methods. (A) One day after electroporation, cells were treated with 20 μM TA for different periods. After incubation, whole cell lysates were prepared, normalized, and used in standard Western blots for the indicated molecules. Note that expression of focal adhesion molecules, but not of control GFP, resulted in the blockade of TA-mediated signaling inactivation and caspase-3 activation. (B) One day after electroporation, cells were treated with DMSO or 20 μM TA for 24 h. After incubation, cells were collected and stained with propidium iodide for DNA content analysis, as above. Data shown represent three independent experiments.

Figure 6. TA interferes with TM4SF5-mediated tumor formation in nude mice. (A and D) SNU449Cp or SNU449T16 (A) or CT26 murine colon carcinoma (D) cells were injected subcutaneously into nude mice (5 x 10^6 cells/mouse). Normal maintenance was performed according to the procedures in the Seoul National University Laboratory Animal Maintenance Manual. Significant tumorigenesis in SNU449T16-injected mice (n=7) or

CT26-injected mice (n=7) was observed. However, SNU449Cp control cell-injected mice (n=7) did not form any significant tumors, and thus no graphic presentation for SNU449Cp was included (A). IP injection of TA at 3 (n=7) or 30 mg/kg body weight (n=7) was performed every other day after the TM4SF5-mediated tumors had been about 200 mm^3. Tumor volume values calculated and body weights measured, as explained in Materials and Methods, were plotted as mean ± standard deviation. (B) Extracts from control tissue or tumor tissue of SNU449T16 cell-injected mice were prepared and immunoblotted for the indicated molecules, as explained in Materials and Methods. (C) Immunohistochemical staining of tumors from SNU449T16 cell-injected mice with DMSO or TA (30 mg/kg in 5% DMSO) administration was performed for active caspase-3, as explained in Materials and Methods. Note that TM4SF5-tissue section with TA administration shows apoptotic cells positive for active caspase-3 in the middle of the tumor mass. (Western blot insert of D) Subconfluent SNU449T16 (T16) and CT26 cells were harvested and immunoblotted for TM4SF5 or α-tubulin to see no endogenous level of TM4SF5 in CT26 cells. Data shown represent three independent experiments.

Figure 1

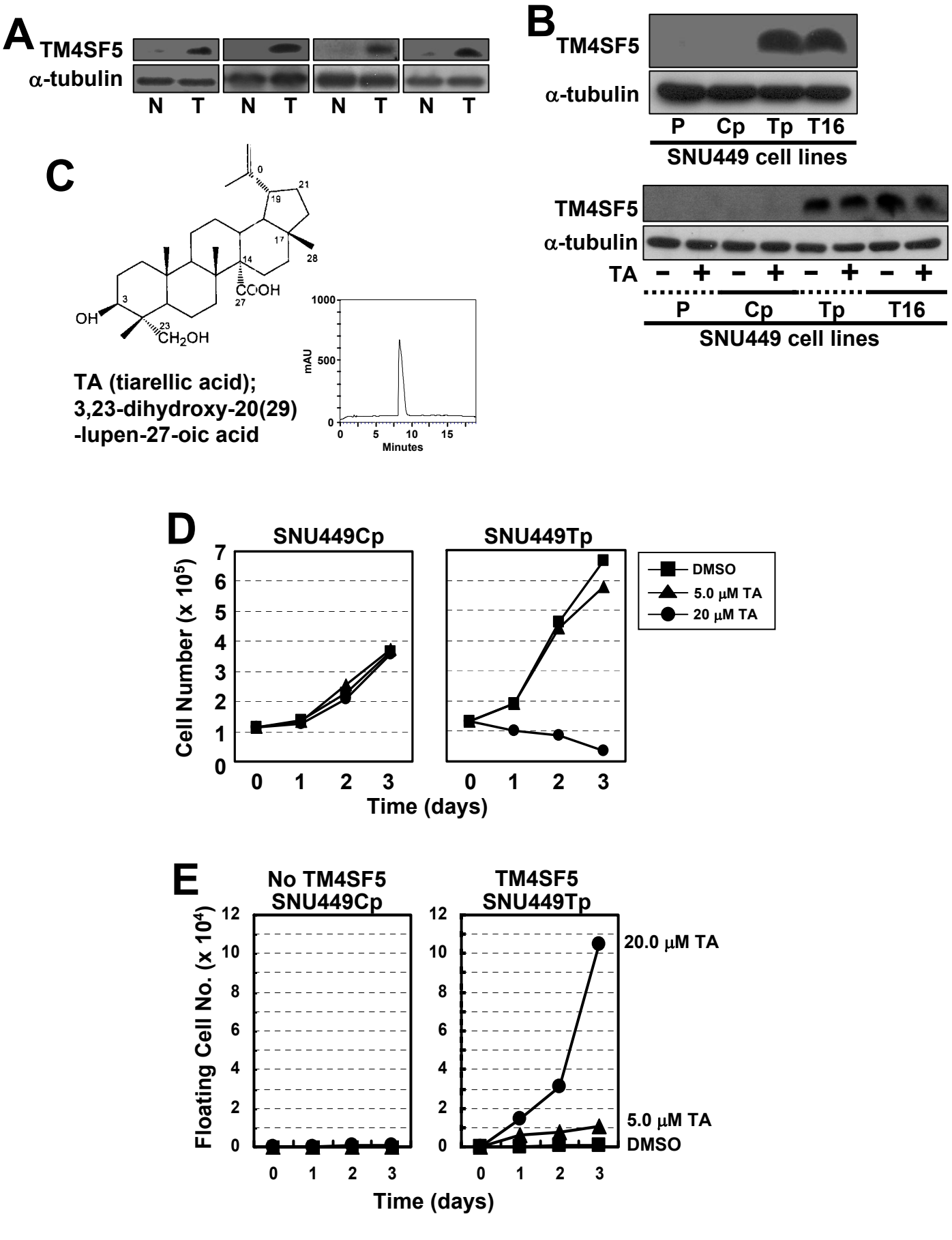
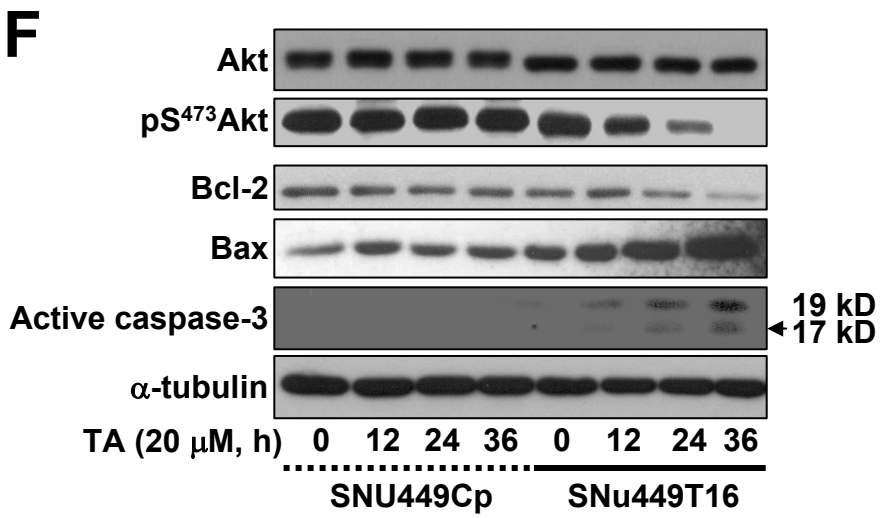

A
TM4SF5
α-tubulin
N T N T N T N T
B
TM4SF5
α-tubulin
P Cp Tp T16
SNU449 cell lines
TM4SF5
α-tubulin
TA - + - + - + - +
P Cp Tp T16
SNU449 cell lines
C
OH
CH2OH
COOH
TA (tiarellic acid);
3,23-dihydroxy-20(29)
-lupen-27-oic acid
mAU
Minutes
D
SNU449Cp
SNU449Tp
Cell Number (x 10^5)
Time (days)
DMSO
5.0 μM TA
20 μM TA
E
No TM4SF5
SNU449Cp
TM4SF5
SNU449Tp
Floating Cell No. (x 10^4)
Time (days)
20.0 μM TA
5.0 μM TA
DMSO

Figure 1


F
Akt
pS473Akt
Bcl-2
Bax
Active caspase-3
19 kD
17 kD
α-tubulin
TA (20 μM, h) 0 12 24 36 0 12 24 36
SNU449Cp
SNu449T16

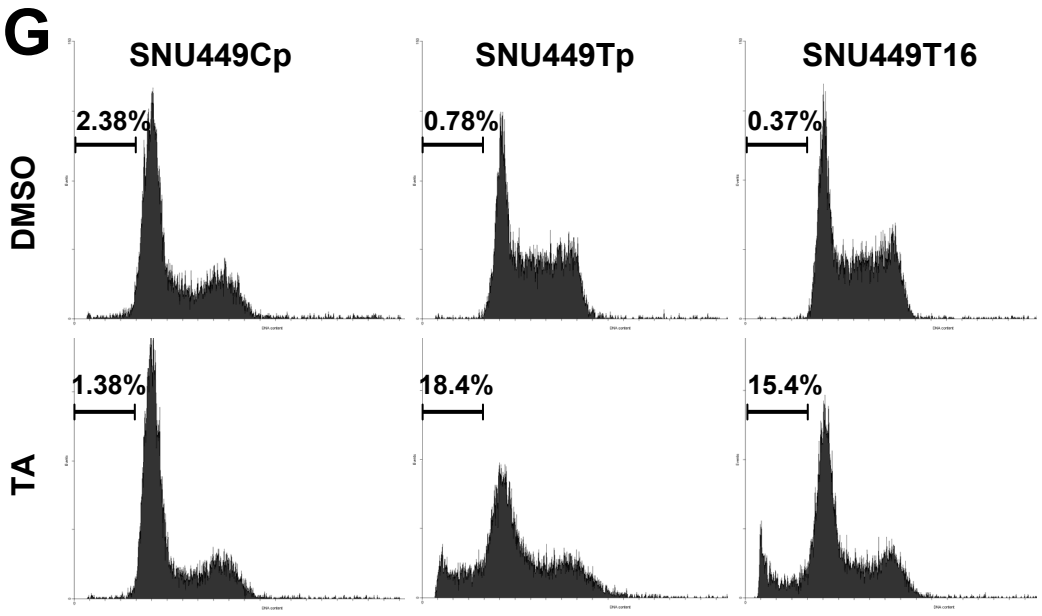

G
SNU449Cp
SNU449Tp
SNU449T16
DMSO
TA
2.38%
0.78%
0.37%
1.38%
18.4%
15.4%

Figure 2

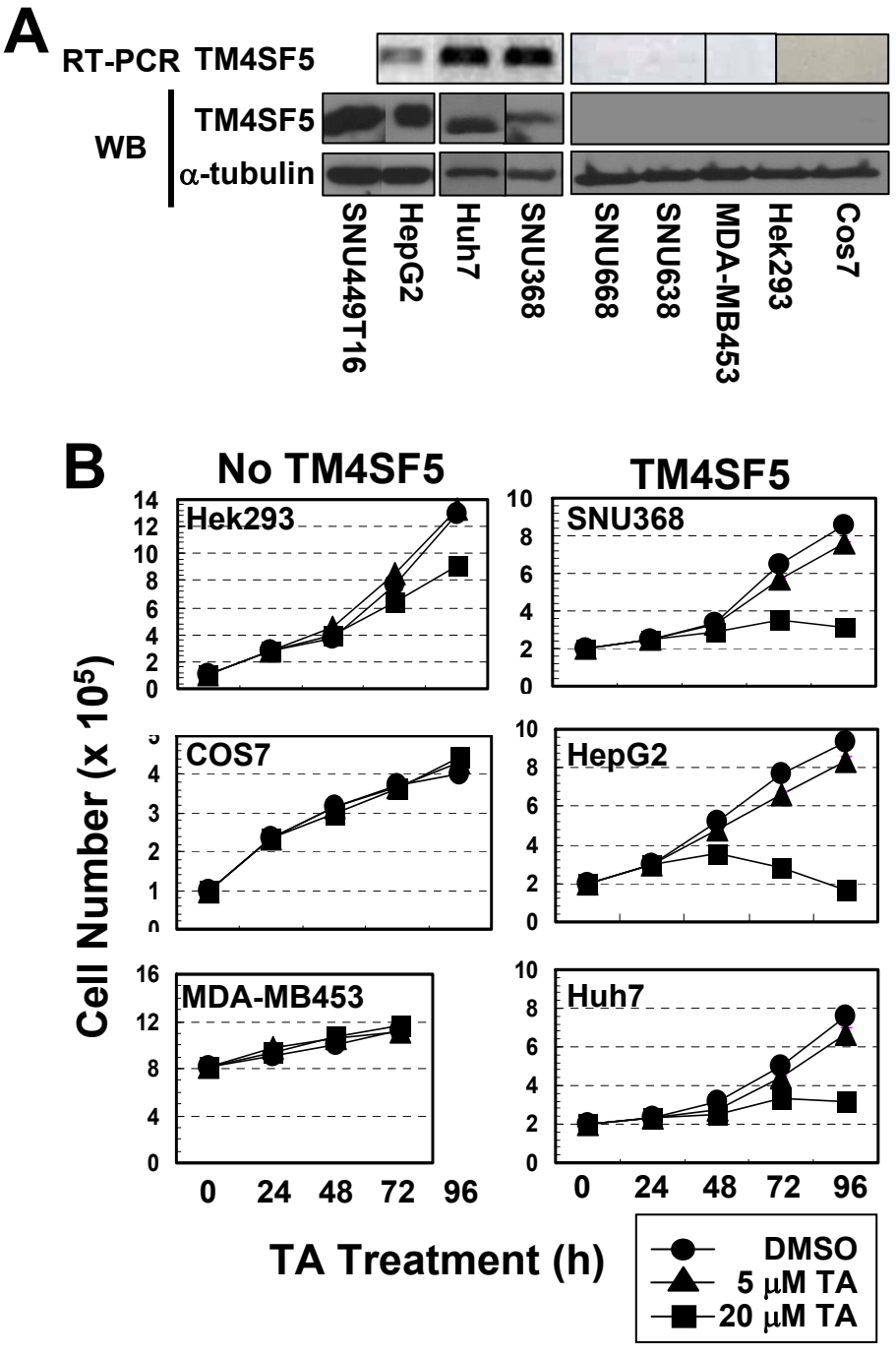

A
RT-PCR TM4SF5
WB
TM4SF5
α-tubulin
SNU449T16
HepG2
Huh7
SNU368
SNU668
SNU638
MDA-MB453
Hek293
Cos7
B
No TM4SF5
TM4SF5
Hek293
SNU368
COS7
HepG2
MDA-MB453
Huh7
Cell Number (x 10^5)
0 24 48 72 96
TA Treatment (h)
DMSO
5 μM TA
20 μM TA

Figure 3

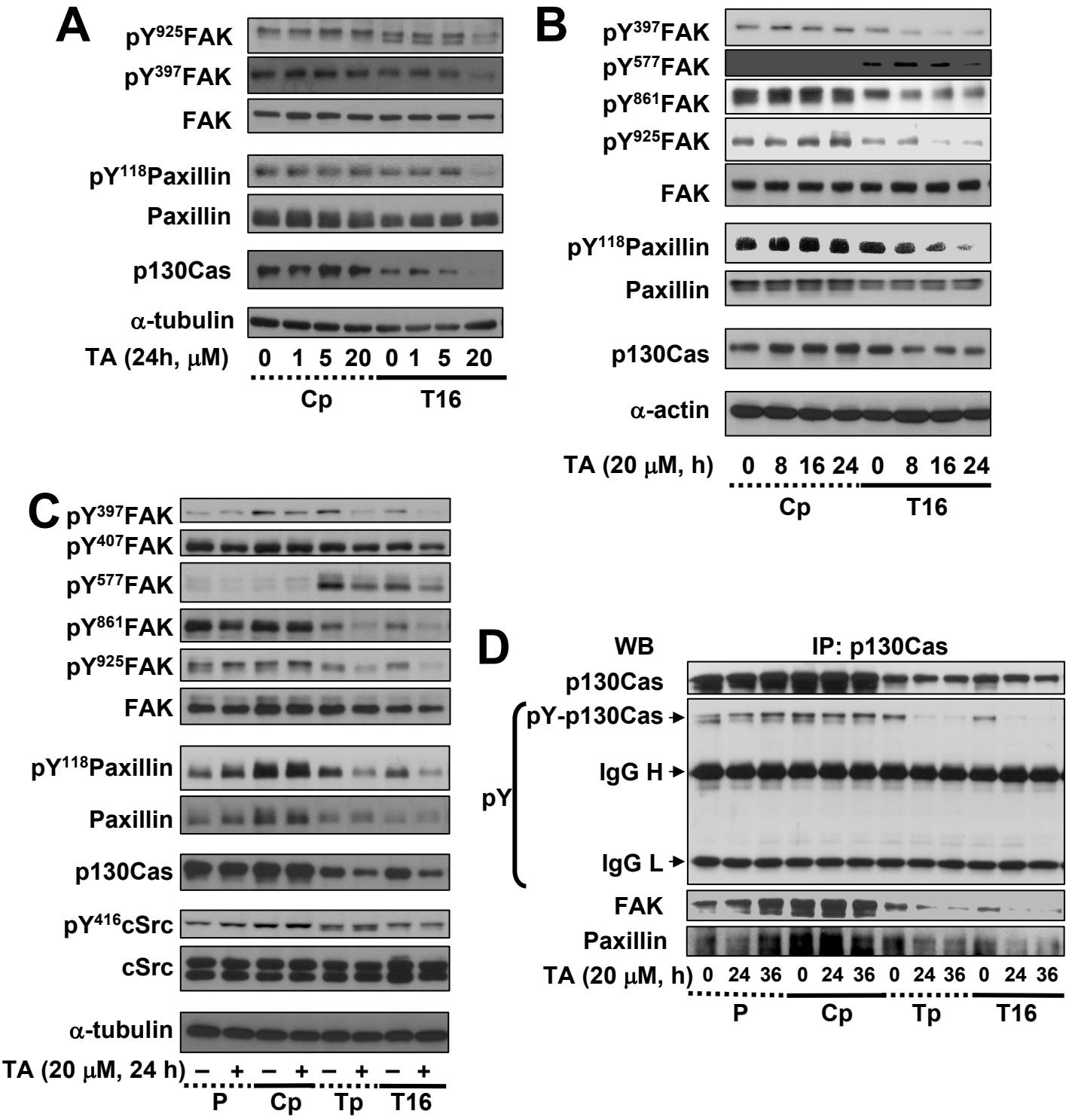

A
pY925FAK
pY397FAK
FAK
pY118Paxillin
Paxillin
p130Cas
α-tubulin
TA (24h, μM) 0 1 5 20 0 1 5 20
Cp
T16
B
pY397FAK
pY577FAK
pY861FAK
pY925FAK
FAK
pY118Paxillin
Paxillin
p130Cas
α-actin
TA (20 μM, h) 0 8 16 24 0 8 16 24
Cp
T16
C
pY397FAK
pY407FAK
pY577FAK
pY861FAK
pY925FAK
FAK
pY118Paxillin
Paxillin
p130Cas
pY416cSrc
cSrc
α-tubulin
TA (20 μM, 24 h) – + – + – + – +
P
Cp
Tp
T16
D
WB
IP: p130Cas
p130Cas
pY-p130Cas
IgG H
IgG L
pY
FAK
Paxillin
TA (20 μM, h) 0 24 36 0 24 36 0 24 36 0 24 36
P
Cp
Tp
T16

Figure 3

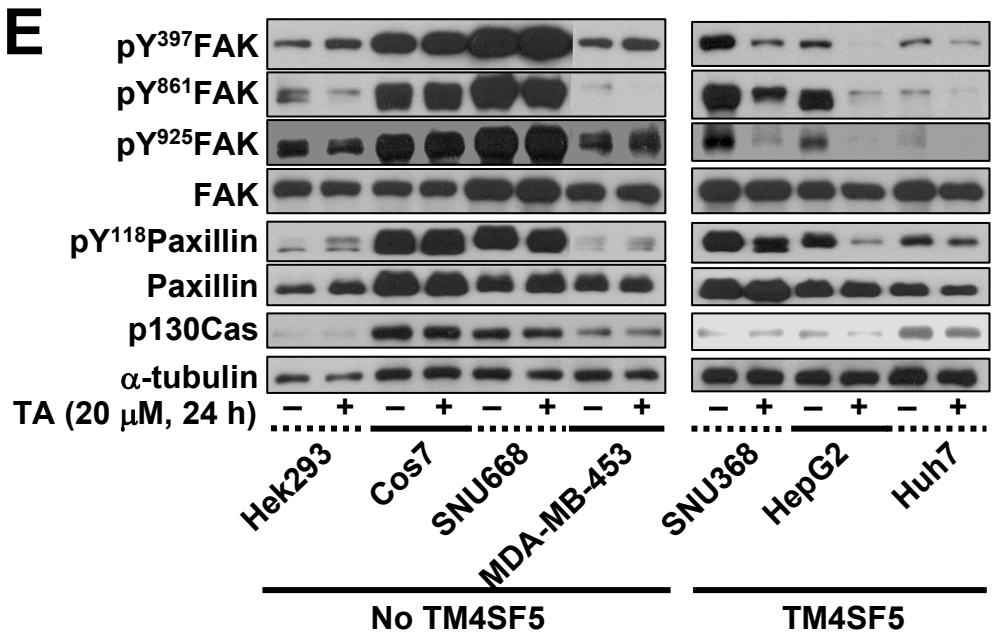

E
pY397FAK
pY861FAK
pY925FAK
FAK
pY118Paxillin
Paxillin
p130Cas
α-tubulin
TA (20 μM, 24 h)
– + – + – + – +
– + – + – +
Hek293
Cos7
SNU668
MDA-MB-453
SNU368
HepG2
Huh7
No TM4SF5
TM4SF5

Figure 4

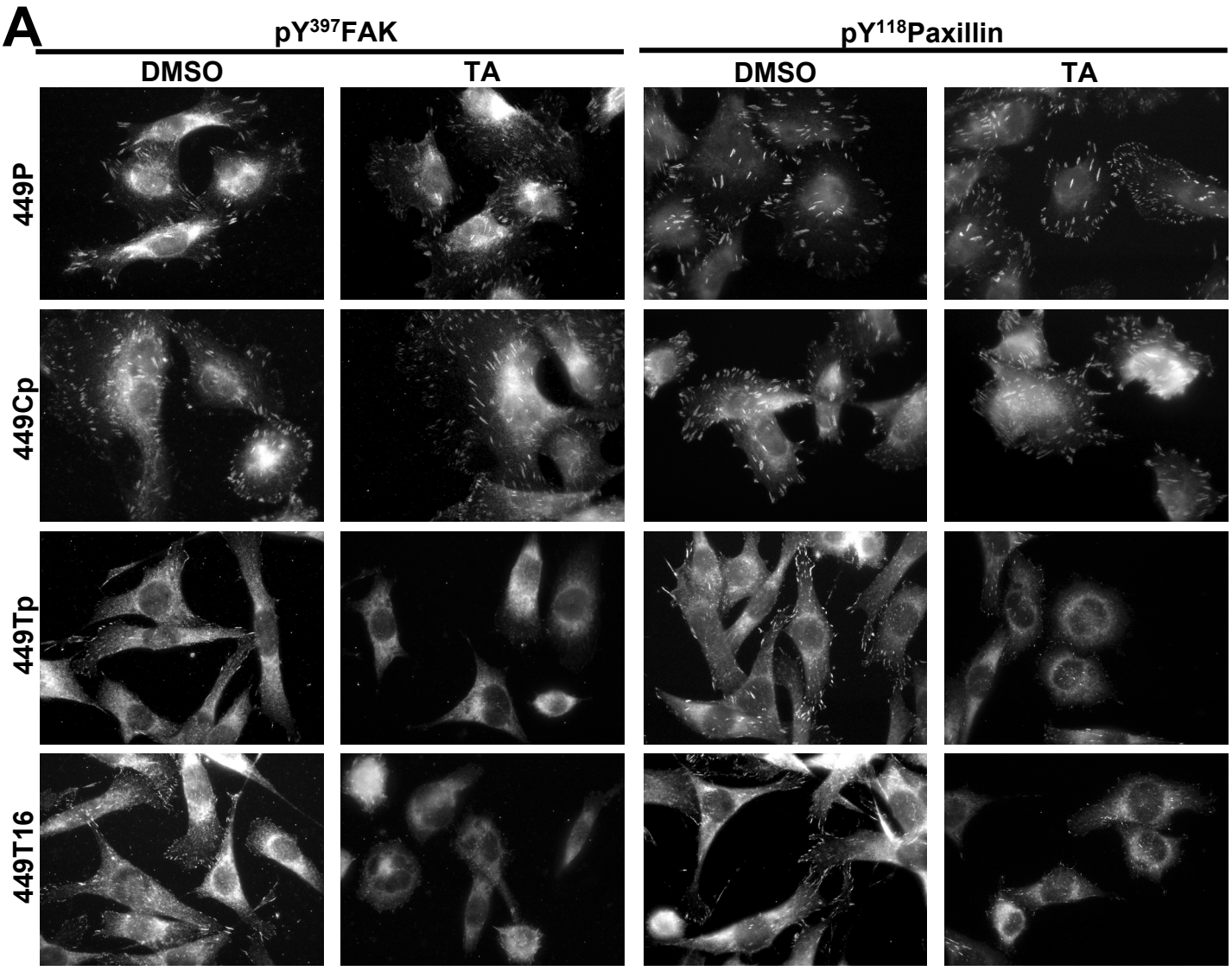

A
pY397FAK
pY118Paxillin
DMSO
TA
DMSO
TA
449P
449Cp
449Tp
449T16

Figure 4

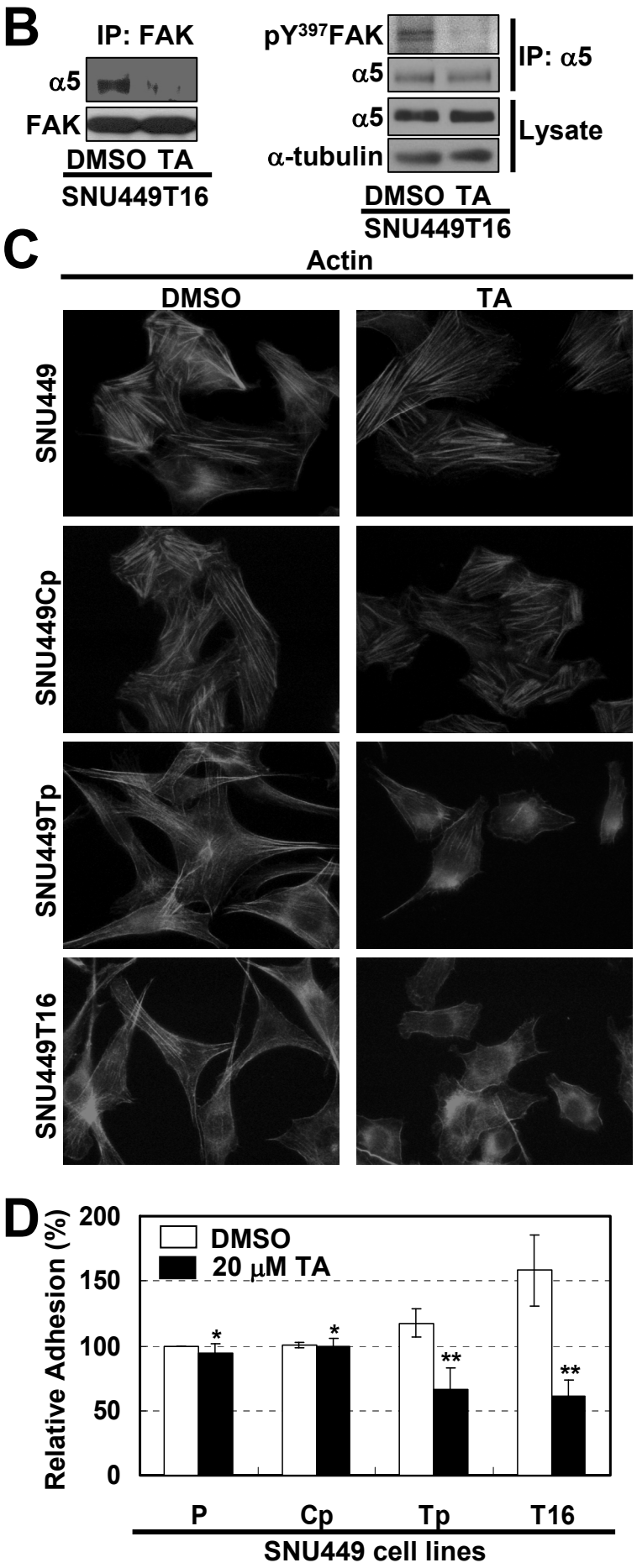
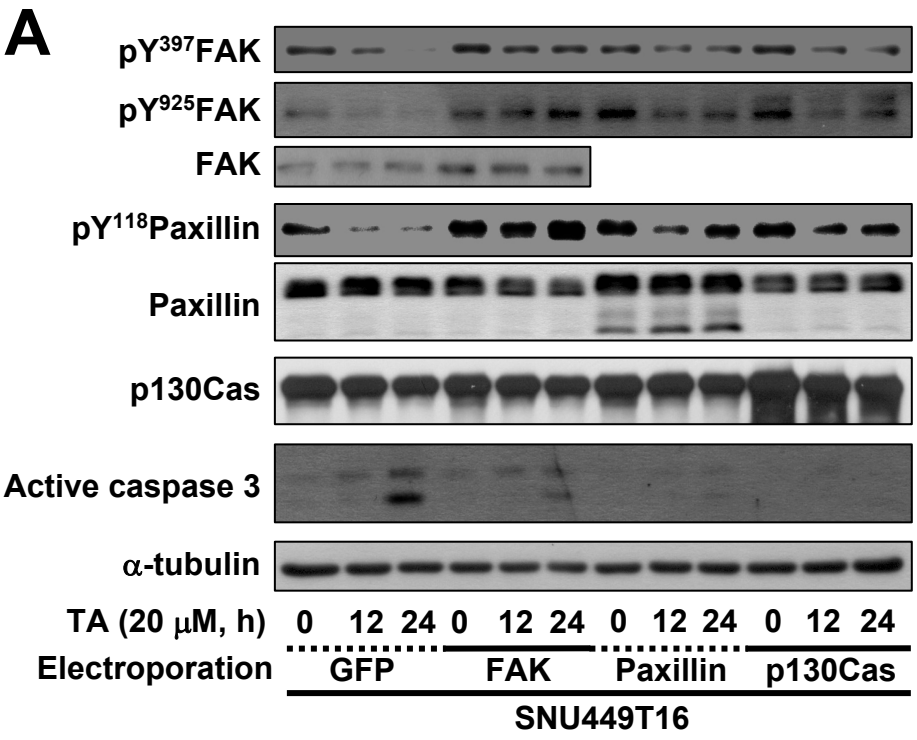

B
IP: FAK
α5
FAK
DMSO TA
SNU449T16
pY397FAK
α5
IP: α5
α5
α-tubulin
Lysate
DMSO TA
SNU449T16
C
Actin
DMSO
TA
SNU449
SNU449Cp
SNU449Tp
SNU449T16
D
Relative Adhesion (%)
200
150
100
50
0
DMSO
20 μM TA
*
*
**
**
P
Cp
Tp
T16
SNU449 cell lines

Figure 5


A
pY397FAK
pY925FAK
FAK
pY118Paxillin
Paxillin
p130Cas
Active caspase 3
α-tubulin
TA (20 μM, h) 0 12 24 0 12 24 0 12 24 0 12 24
Electroporation GFP FAK Paxillin p130Cas
SNU449T16

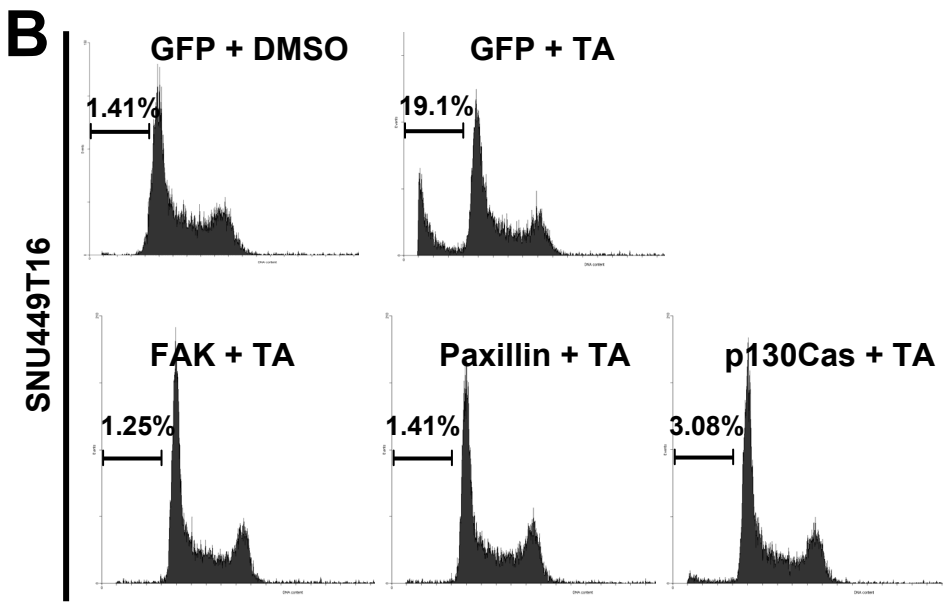
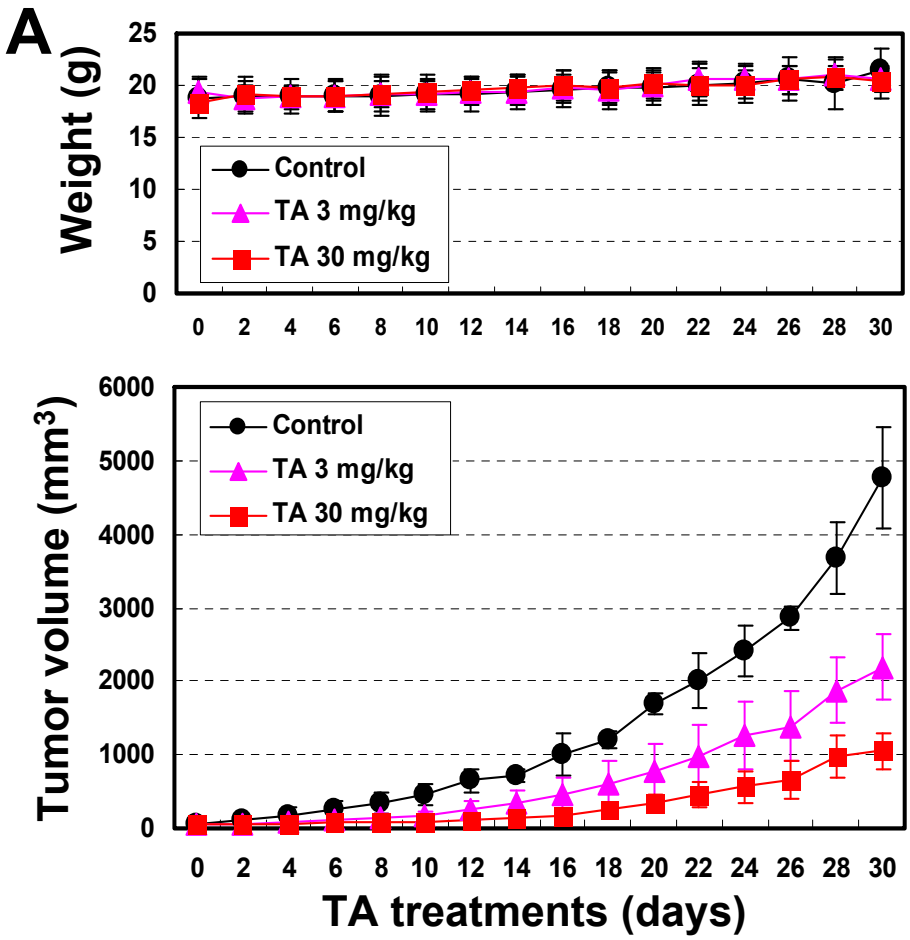
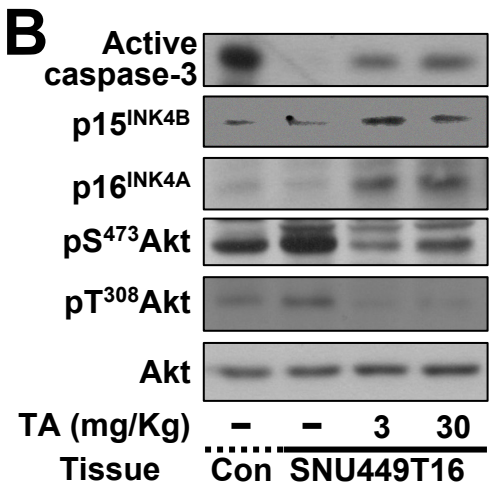

B
SNU449T16
GFP + DMSO
1.41%
GFP + TA
19.1%
FAK + TA
1.25%
Paxillin + TA
1.41%
p130Cas + TA
3.08%

Figure 6


A
Weight (g)
25
20
15
10
5
0
Control
TA 3 mg/kg
TA 30 mg/kg
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Tumor volume (mm3)
6000
5000
4000
3000
2000
1000
0
Control
TA 3 mg/kg
TA 30 mg/kg
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
TA treatments (days)


B
Active caspase-3
p15INK4B
p16INK4A
pS473Akt
pT308Akt
Akt
TA (mg/Kg) – – 3 30
Tissue Con SNU449T16

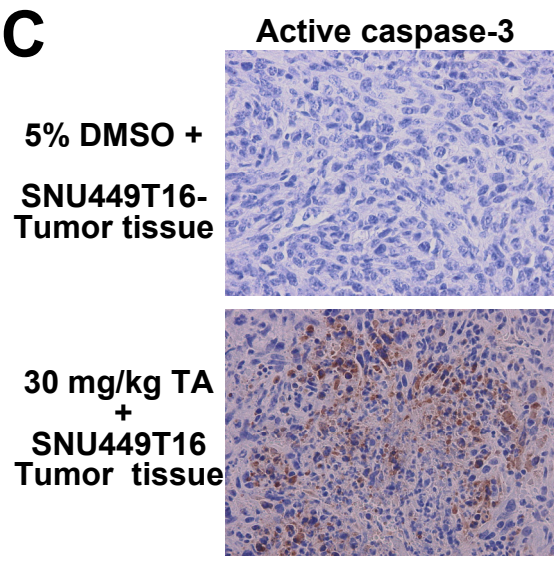
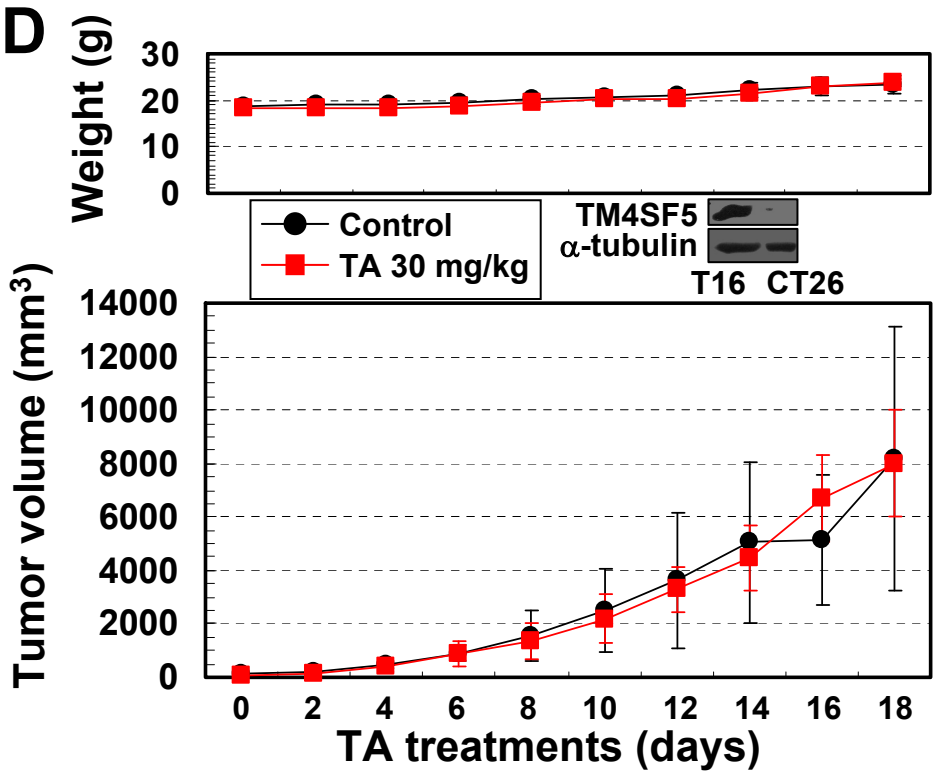

C
Active caspase-3
5% DMSO +
SNU449T16-
Tumor tissue
30 mg/kg TA
+
SNU449T16
Tumor tissue

Figure 6


D
Weight (g)
30
20
10
0
Control
TA 30 mg/kg
TM4SF5
α-tubulin
T16
CT26
Tumor volume (mm3)
14000
12000
10000
8000
6000
4000
2000
0
0
2
4
6
8
10
12
14
16
18
TA treatments (days)