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A Requirement for ZAK Kinase Activity in Canonical TGF-β Signaling[1]

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Abstract

The sterile alpha motif and leucine zipper containing kinase ZAK (AZK, MLT, MLK7), is a MAPK-kinase kinase (MKKK). Like most MAPKKKs which are known to activate the c-Jun. amino-terminal kinase (JNK) pathway, ZAK has been shown to participate in the transduction of Transforming growth factor-β (TGF-β)-mediated non-canonical signaling. A role for ZAK in SMAD-dependent, canonical TGF-β signaling has not been previously appreciated. Using a combination of functional genomics and biochemical techniques, we demonstrate that ZAK regulates canonical TGFβRI/II signaling in lung and breast cancer cell lines and may serve as a key node in the regulation of TGFBR kinase activity. Remarkably, we demonstrate that siRNA mediated depletion of ZAK strongly inhibited TGF-β dependent SMAD2/3 activation and subsequent promoter activation (SMAD binding element driven luciferase expression; SBE4-Luc). A ZAK specific inhibitor (DHP-2), dose-dependently activated the bioluminescent TGFBR-kinase activity reporter (BTR), blocked TGF-β induced SMAD2/3 phosphorylation and SBE4-Luc activation and cancer cell-invasion. In aggregate, these findings identify a novel role for the ZAK kinase in canonical TGF-β signaling and an invasive cancer cell phenotype thus providing a novel target for TGF-β inhibition.

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Introduction

Transforming growth factor-β (TGF-β) is an evolutionarily conserved superfamily of cytokines that regulate many cellular functions in a context dependent manner. TGF-β signals through a heteromeric receptor complex consisting of type I (TGFBR1) and type II (TGFBR2) serine/threonine kinase receptors. TGF-β binding to TGFBR2 leads to its phosphorylation and subsequent recruitment and activation of TGFBR1. Activated receptor complex subsequently recruits and phosphorylates receptor regulated smads (R-Smads). R-smads (SMAD2/3) form a complex with a co-smad (SMAD4) and translocate to nucleus where they regulate transcriptional responses. TGF-β also activates other signaling cascades, including MAPK pathways. Ligand-dependent activation of the TGF-β receptors and regulation of their subsequent kinase activity is a complex process involving multiple positive and negative regulatory mechanism [1,2]. ZAK was identified in a human kinome siRNA screen utilizing a live cell reporter for TGFBR1 kinase activity (BTR; [3,4]).

The sterile alpha motif and leucine zipper containing kinase ZAK (AZK, MLT, MLK7) is a MAP kinase kinase kinase. These mixed lineage kinases (MLKs) cluster into three subgroups based on the domain arrangements and sequence similarity within their catalytic domains: the MLKs, the dual-leucine-zipper-bearing kinases, and zipper sterile-α-motif kinase (ZAK). ZAK is an 800 amino acids protein that contains a kinase catalytic domain, a leucine zipper, and a sterile-a-motif (SAM). ZAK has two isoforms, the larger one (ZAK-α or MLK-like MAP triple kinase-α; MLTK-α) and an alternative splicing product (ZAK-β, MLTK-β or MLK7) which lacks a SAM domain

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[5,6]. Similar to other MLK family members, the MLTKs regulate signaling of the extracellular signal-regulated kinase (ERK), c-Jun, N-terminal kinase (JNK), and p38 kinase.

ZAK expression in mammalian cells has been shown to regulate JNK/SAPK pathway and NF-κB transcription factor activation [7]. MLTK-α overexpression has been shown to enhance anchorage independent growth in response to EGF [8]. ZAK has been shown to inhibit growth of lung cancer cells via ERK and JNK activation in an AP-1 dependent manner [9]. ZAK has also been shown to be a critical component of TGF-β dependent hypertrophic growth in cardiomyoblast cells [10]. Over-expression of a dominant-negative mutant of ZAK was shown to abolish TGF-β induced features of cardiac hypertrophy, including increased cell size, higher expression of atrial natriuretic factor (ANF) and increased organization of actin fibers [10]. Although these data implicate ZAK in non-canonical TGF-β signaling, its role in canonical TGF-β signaling in cancer is unclear.

We herein describe a role for ZAK in TGF-β mediated canonical (SMAD2/3) signaling. siRNA mediated depletion of ZAK or its inhibition using DHP-2 resulted in activation of a reporter for the TGFBR1 kinase activity, attenuated SMAD2/3 activation as well as downstream transcriptional activity (SBE4-Luc), and an invasive cellular phenotype. We further show that the interaction of ZAK to TGFBR1 is independent of ligand mediated activation as well as SB-431542 mediated inhibition of TGFBR1 kinase activity. Taken together, our data show that ZAK kinase activity plays an important role in canonical TGF-β signaling thus providing an additional node for the regulation of this key oncogenic pathway.

Materials and Methods

Plasmid DNA

BTR WT reporter has been described earlier [3]. SBE4-Luc [11] reporter plasmid was provided by Dr. Bert Vogelstein (Addgene plasmid #16,527). pCMV5-TGFBRI-His (#19,161) was provided by Dr. Joan Massague [12]. ZAK cDNA clone was obtained from Harvard DNA repository and cloned into pEF vector backbone expressing 3XFL-tag. ZAK dominant negative (ZAK-dn; Lys45Met [13]) and constitutively active ZAK (ZAK-EE; Ser230/234Glu [13]) were created by site directed mutagenesis.

Antibodies and Reagents

Antibodies to pSMAD2, SMAD2, pSMAD3, TGFBR1, c-JUN, pERK1/2 ERK1/2 and GAPDH (all from Cell Signaling), SMAD3 (Invitrogen), TGFBR1 (V22), and SARA (Santa Cruz biotechnology), firefly luciferase (Millipore), Flag-HRP (Sigma), His-tag-HRP (clone H3; Invitrogen), and His-tag (clone H8; Millipore), ZAK monoclonal antibody MO3 (clone 3G5; Abnova) and ZAK polyclonal antibody was from Novus Biologicals. The HRP-conjugated secondary antibodies were from Jackson ImmunoResearch. Recombinant human TGF-β1 was obtained from HumanZyme, Alkaline phosphatase was purchased from New England Biolabs (NEB). TGFBR1 inhibitors SB-431,542 and SD-208 were obtained from Cayman Chemical and D-Luciferin from Xenogen Corp. ZAK siGENOME Smart Pool siRNA as well as non-silencing siRNA (NSS) were obtained from GE-Dharmacon. The small molecular weight inhibitor of ZAK (DHP-2) was provided by Laura J Bloem from Eli Lilly [14].

Cell Culture and Transfection

The human lung carcinoma cell line A549 and normal kidney cell line HEK293T were obtained from American Type Culture collection (ATCC) maintained in RPMI-1620 or DMEM media respectively supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, and 0.1% penicillin/streptomycin/gentamycin (GIBCO-Invitrogen). Cells were grown in a humidified incubator at 37 °C and 5% CO_2. Breast cancer cell line 1833 [15] derived from MDA-MB-231 was kindly provided by Dr. Joan Massague (Memorial Sloan Kettering Institute, NY) and maintained in DMEM media in the same conditions mentioned above. A549 and MDA-231-1833 cell lines stably expressing the BTR reporter were previously generated [3] and maintained as described. siGENOME siRNA pool were transfected using Dharmafect1 while plasmids were transfected using Fugene6.

Western Blot Analysis

Western analysis was carried out using standard protocols. Cells were grown in culture dishes, transfected with specific siRNA or plasmids, or treated with select compounds and TGF-β for designated time periods, and cell lysates were resolved on SDS-PAGE gels and transferred to PVDF membranes. Membranes were probed against specific primary antibodies followed by HRP conjugated secondary antibodies then visualized using the Enhanced Chemiluminescence (ECL) Western Blotting System (GE Healthcare). Signal intensity was measured using an image processing and analysis program (ImageJ, v1.45) [16].

Co-Immunoprecipitation

For co-immunoprecipitation studies HEK293T cells were transfected with the indicated plasmids in 10 cm plates. Cells were treated with TGF-β and inhibitors for the indicated time and lysates were made 48–72 h post-transfection in IP-lysis buffer (50 mM Tris PH 7.4, 1% NP40, 0.25% Deoxycholate sodium salt, 150 mM NaCl, 10% Glycerol, and 1 mM EDTA) supplemented with 1X PhosStop (Roche), 1X Protease inhibitor cocktail (Roche), Sodium Ortho Vanadate, Sodium fluoride, PMSF, and β-Glycerol phosphate (2 μM each). Protein estimation was performed using detergent compatible Dc assay kit (Pierce). Lysates were pre-cleared by incubating with normal Rabbit-IgG for 1 h followed by 30 μl protein A/G-coupled Sepharose beads (GE Healthcare) for an additional hour at 4 °C and centrifuged. Co-immunoprecipitation was carried out by incubating pre cleared cell lysate (400 μg protein) with 1.5 μg TGFBR1 or ZAK specific polyclonal antibodies overnight at 4°C. The immune complex was captured using 30 μl slurry of protein A/G-coupled Sepharose beads for 2 h, washed four times with lysis buffer. For the Flag-tag IP, lysates were pre-cleared with normal mouse IgG- and protein A/G-coupled Sepharose beads and the immunoprecipitation was carried out using Flag-M2 beads (Sigma) for 2 hours at room temperature. The resulting pellet was resolved by SDS-PAGE and transferred to PVDF membrane for western-analysis.

BTR Reporter Assay

A549-BTR (5×10^3 cells/well) and MDA-231-1833-BTR (6×10^3 or cells/well) cell lines were seeded in 96-well black-walled, clear-bottom plates (Corning, Inc., Corning, NY) 24 to 48 hours prior to assaying. Cells were treated in serum-free media with various concentrations of DHP-2 or SD-208 for indicated time periods. Saturating concentration of the firefly luciferase D-Luciferin (100 μg/ml final concentration) were added to the media 5 minutes before starting the live-cell luminescence imaging. Photon counts for each condition were acquired for 60 seconds at medium binning using an IVIS 200 imaging system (Xenogen-PerkinElmer, Alameda, CA) for 15 sequences for each time point.

SBE4-Luc Reporter Assay

For SBE4-Luc reporter studies, 1.25 X 10^5 MDA-231-1833 cells were plated in 12 well plates 1 day prior to transfection. Cells were transfected next day with 100 ng SBE4-Luc plasmid, 100 nM siRNA, and 20 ng Gaussia-Luciferase (internal control for transfection efficiency) for each well with Lipofectamine 2000. Cells were serum starved 24 h post transfection for 6 h and treated with 10 ng/mL TGF-β for 24 h before measuring SBE4-Luc bioluminescence activity. Alternately, cells were treated with 10 μM SB-431,542 or DHP-2 in the presence of TGF-β. Cells were washed twice with PBS and kept in fresh media for 8-24 h before measuring Gaussia-Luc activity.

Hanging Drop Cell Invasion Assay

The invasive nature of cancer cell lines as a correlate of metastatic potential in a biologically relevant three dimensional format was evaluated using the cell spheroid invasion assay [17]. Gravity was used to generate spheroids within drops of media that hang from the lid of a cell culture dish. Next, these spheroids are embedded in a 3D matrix consisting of a mixture of basement membrane materials and type I collagen. Cancer cell egression from the spheroids into the surrounding matrix was then monitored over time.

Results

ZAK Regulates Canonical TGF-β Signaling

In an effort to identify novel regulators of TGF-β dependent Smad activation, we utilized a protein complementation reporter for SMAD2 targeted TGFβR1 kinase activity (BTR) [3]. TGF-β-responsive lung adenocarcinoma cells (A549) as well as a metastatic variant breast cancer cell line (MDA-231-1833, [18]) were engineered to stably express the reporter and utilized in a high-throughput siRNA screen against the Human Kinome library. This screen led to the identification of ZAK among several other kinases as regulators of canonical TGF-β signaling [4]. Although a role of ZAK in non-canonical TGF-β signaling (MAPK) and its interaction with TGFBR1 have been described [10], a role for ZAK in canonical (SMAD2/3 dependent) TGF-β signaling has not been previously appreciated. Studies wherein siRNA mediated knock down of ZAK in BTR expressing cells resulted in an increase in the BTR activity compared to non-targeted scrambled siRNA (NSS) cells validated the screen (Figure 1, *A* and *B*). Biochemical confirmation was accomplished by ZAK specific siRNA knockdown followed by an over-night serum starvation and treatment with TGF-β for 1 hour. Analysis of SMAD2-phosphorylation from the resulting cell extracts revealed a decrease in phospho-SMAD2 levels when ZAK expression was knocked out, whereas no change in the level of total SMAD2 was observed (Figure 1*C*). Similarly SMAD3 phosphorylation was also reduced in ZAK knockdown cells (Supplementary Figure 1*A*). This data indicates that ZAK is required for TGF-β mediated SMAD2/3 activation (canonical TGF-β signaling).

SMAD2/3 phosphorylation in response to TGF-β leads to their translocation to the nucleus, leading to transcriptional activation of target genes [19]. To investigate if TGF-β dependent transcriptional regulation is also affected by ZAK, MDA-231-1833 cells were transfected with a Smad-binding element driven luciferase reporter plasmid (SBE4-Luc [20],) in the presence or absence of ZAK knockdown. Overnight stimulation of cells using TGF-β, resulted in an increase in SBE4-Luc activity in NSS transfected cells, whereas cells wherein ZAK was knocked down had a diminished response to TGF-β stimulation (Figure 1*D*). Immunoblot analysis of the lysates revealed a decrease in SBE4-Luc derived luciferase expression in the presence of ZAK knockdown (Figure 1*E*) compared to control siRNA-transfected cells, further confirming results obtained from luciferase activity assays.

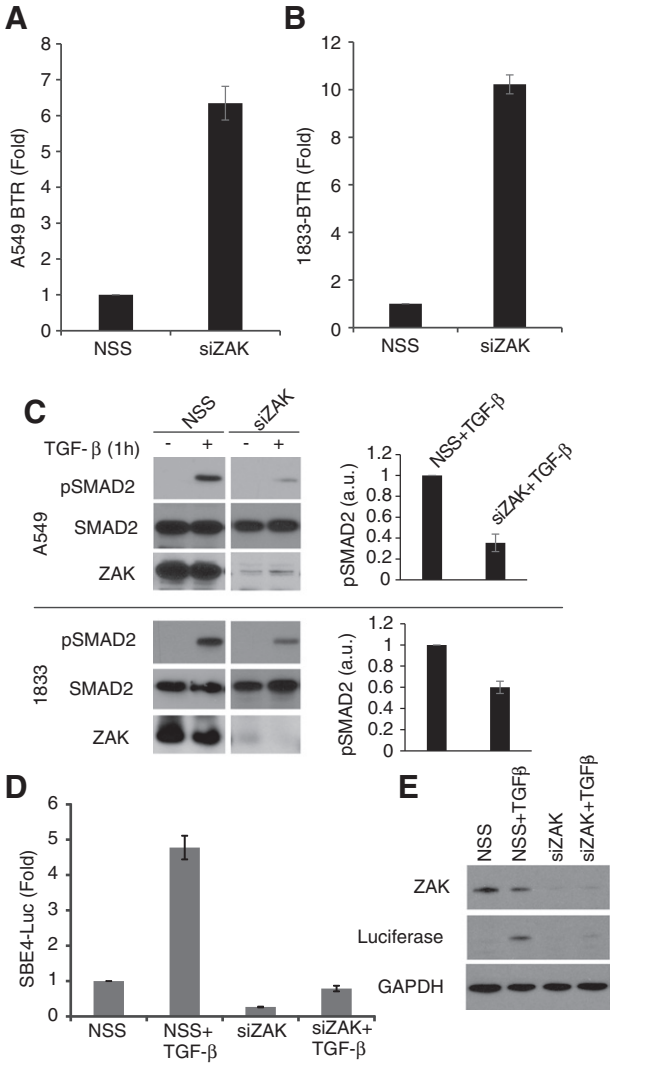

Figure 1. ZAK mediates TGF-β dependent SMAD2 phosphorylation. The Bioluminescent TGF-β reporter expressing lung (A549-BTR; A) and breast (1833-BTR; B) cancer cell lines were transfected nonspecific scrambled control (NSS, negative control) or ZAK specific siRNA and bioluminescence was measured 72 hours post transfection. Bar graphs are mean of at least three biological replicates. Error bars represent ±SD. In parallel experiments, A549 and MDA-231-1833 cells were transfected with control or ZAK specific siRNA, serum starved, treated with 5 ng/mL TGF-β for 1 hour. Extracts were prepared and analyzed for phosphorylated SMAD2 and SMAD2 abundance by immunoblot analysis (C). ZAK immunoblotting was performed to confirm the knockdown efficiency. Western-blot quantitation was performed using Image J. Bar graphs are mean absorbance units (AU) of at least three biological replicates (TGF-β treated samples only). Error bars represent ±SD. Relative firefly luciferase activity (normalized to Gaussia luciferase) in MDA-231-1833 (D) cells transiently transfected with the SBE4-Luc reporter plasmid and a GLuc plasmid and treated with 10 ng/mL TGF-β for 24 hours. Data are means ± S.E.M. of 3 biological replicates. Total cellular extracts were prepared in parallel experiments and probed with luciferase and ZAK specific antibodies to confirm the transfection efficiency and treatment (E). GAPDH was used as a loading control.

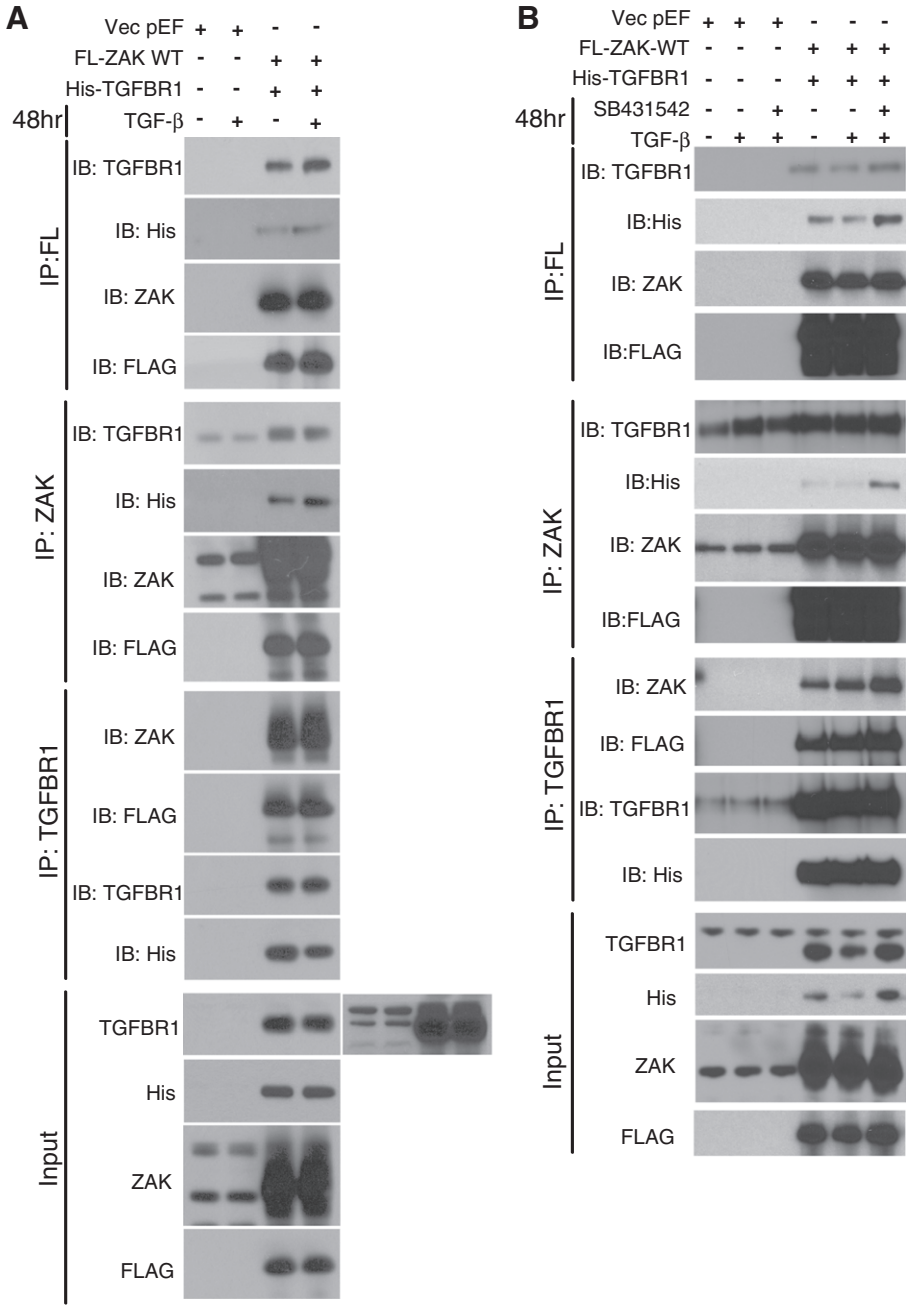

Figure 2. ZAK interaction to TGFBR1 is independent of TGFBR1 kinase activity. (A) HEK293T cells were transfected with FL-ZAK and His-TGFBR1 plasmids and treated with 10 ng/mL TGF-β for 48 hours. Total cellular extracts were prepared and immunoprecipitation was performed using ZAK and TGFBR1 antibodies or FL antibody conjugated sepharose beads. Immunoblots were performed as labeled. (B) HEK293T cells were transfected and treated as described above except that an additional sample was prepared wherein an inhibitor to TGFBR1 kinase activity (SB-431,542; 10 μM) was also used. Immunoprecipitation and immunoblotting was carried out as described above.

Based on the mRNA expression data (Oncomine.org) ZAK is in the top 10% of over-expressed genes in colorectal, gastric, head & neck cancers and in lymphoma, while it is under-expressed in breast, bladder and lung cancers (representative data in Supplementary Figure 1, *B* and *C*). Furthermore, to investigate whether overexpression of ZAK had any effect on autocrine TGF-β signaling or downstream effector molecules, we overexpressed wild type (WT, kinase active), dominant negative (kinase inactive, dn, [13]) as well as a constitutively active (ZAK-EE) mutant of ZAK in HEK293T cells (Supplementary Figure 1*D*). Transfected cells were serum starved and treated with TGF-β in the presence or absence of SB431542. Resulting lysates were resolved on SDS-PAGE gels. In contrast to empty vector transfected cells, over expression of ZAK-WT did not potentiate TGF-β response as observed by similar activation of SMAD2. However, over expression of WT and constitutive active ZAK led to markedly increased activation of a TGF-β responsive gene c-Jun. as well as ERK1/2 (Supplementary Figure 1*D*).

ZAK Interacts With TGFBR1 in a Ligand Independent Manner

To provide biochemical support for the above findings, we investigated if ZAK interacted with TGFBR1 and whether this interaction was dependent on TGFBR1 kinase activity. HEK293T

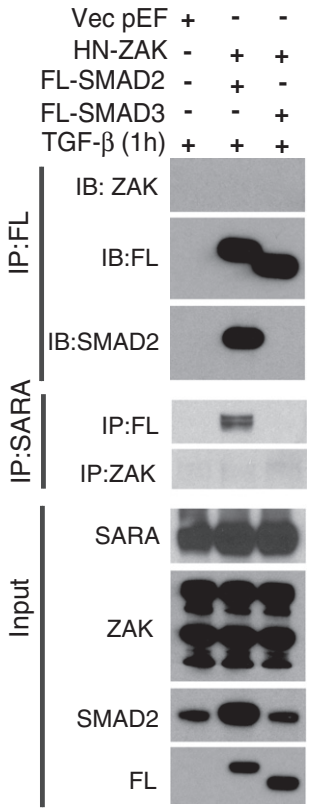

Figure 3. ZAK does not interacts with SMAD2/3 or SARA. HEK293T cells were transfected with FL-SMAD2, FL-SMAD3 and HN-ZAK and treated with TGF-β for 1 hour. Resulting lysates were immunoprecipitated with FL-sepharose or SARA-specific antibodies and probed with antisera against ZAK, FL, SARA, and SMAD2.

cells were transfected with FLAG-tagged ZAK (FL-ZAK) and Histidine-tagged TGFBR1 (His-TGFBR1). Stimulation of transfected cells with TGF-β for 48 hours followed by immunoprecipitation of the resulting lysates using antibodies against Flag-tag, ZAK or TGFBR1, revealed similar interaction of ZAK to TGFBR1 in response to TGF-β treatment (Figure 2*A*). Furthermore, parallel studies wherein cells were treated with SB-431,542 revealed that inhibition of TGF-β-receptor kinase activity did not significantly impact the interaction of ZAK with TGFBR1 (Figure 2*B*).

Based on the above finding that ZAK was required for TGF-β dependent SMAD2 phosphorylation, we investigated if ZAK interacts with SARA and SMAD2/3, key components of the canonical cascade. HEK293T cells were transfected with FL-SMAD2 or FL-SMAD3 as well as HN-ZAK, overnight starved cells were then treated with TGF-β ligand for 1 hour and lysates were prepared for immunoprecipitation using antibodies against Flag-tag or SARA. The resulting pellets were probed with a ZAK specific antibody as well as Flag-tag, SMAD2 or SARA specific antibodies. No interaction of ZAK with SMAD2/3 or SARA was detected (Figure 3), although as a positive control interaction of SMAD2 with SARA was readily detected (Figure 3).

ZAK Inhibitor DHP-2 Blocks Canonical TGF-β Signaling

DHP-2 (7-[3-fluoro-4-aminophenyl-(4-(2-pyridin-2-yl-5,6-dihydro-4H-pyrrolo[1,2-b]pyrazol-3-yl))]-quinoline), an inhibitor of ZAK [14,21,22] was utilized to further delineate the role of ZAK-kinase activity in canonical TGF-β signaling. A549 or MDA-231-1833 cells expressing the BTR reporter were treated with TGF-β in the presence of various concentrations of DHP-2 and imaged for BTR activity. Inhibition of ZAK kinase activity resulted in a dose dependent increase in bioluminescence (Figures 4, *A* and *B* and 5, *A* and *B*) indicative of inhibition of canonical TGF-β signaling. Parallel experiments revealed that the IC_{50} for DHP-2 mediated BTR activation was 1.66 μM and 1.56 μM for A549 and MDA-231-1833 respectively (Figures 4*C* and 5*C*). The efficacy of DHP-2 was similar to SD-208, a TGFBR-inhibitor (Figures 4*C* and 5*C*).

To confirm that inhibition of ZAK kinase activity using DHP-2 resulted in abrogation of TGF-β signaling, A549 cells were treated with various concentrations of DHP-2 in the presence of TGF-β for 1 hr. The resulting lysates were probed using a phospho-SMAD2 as well as a phospho-SMAD3 specific antibody (Figure 5*D*). A robust decrease in TGF-β stimulation mediated phosphorylation of SMAD2 as well as SMAD3 was observed in the presence of DHP-2 at sub-micromolar concentrations (Figure 5*D*). There was no apparent change in the levels of total SMAD2/3, TGFBR1, ZAK or luciferase (reporter). Since ZAK is a MAP kinase kinase kinase and is known to play a role in ERK1/2 activation, we additionally carried out western blotting using pP38MAPK and pERK1/2 antibodies to demonstrate that DHP2 at the used concentrations efficiently inhibited ZAK kinase activity (Figure 5*D*).

To further confirm a role for ZAK kinase activity in TGF-β dependent canonical signaling and its resulting transcriptional response, we utilized the SBE4-Luc assay. MDA-231-1833 cells were transfected with the SBE4-Luc plasmid and stimulated with TGF-β in the presence or absence of DHP-2 as well as the TGFBR1 inhibitor (positive control, SB-431542) (Figure 4*D*). In agreement with the decrease in SMAD2/3 phosphorylation observed above, DHP-2 also inhibited the Smad-dependent transcriptional response with an efficacy similar to SB-431542 at 10 μM.

Since the transcriptional response to TGF-β leads to enhanced cancer cell migration and invasion, we investigated if ZAK kinase activity played a role in these cellular phenotypes. MDA-231-1833 cells were allowed to form hanging drop colonies in soft agar [23] and treated with TGF-β alone or TGF-β in the presence of various concentrations of SB-431542 or DHP-2 and allowed to invade for 96 hours (Figure 6, *A* and *B*). As shown in Figure 6*A*, TGF-β stimulation resulted in enhanced cell migration which was significantly inhibited in the presence of DHP-2. In contrast, SB-431542 seemed to reduce the migration of large groups of cells, the migration of single cells was not completely inhibited even at the maximum concentration tested (10 μM; Figure 6*B*).

DHP-2 Inhibits Dimerization of ZAK

Overexpression of ZAK results in distinct higher molecular weight bands which have been shown to be due to its autophosphorylation [10,24] and dimerization [13]. We overexpressed wild type (WT, kinase active) as well as a dominant negative (kinase inactive, dn, (13)) mutant of ZAK in HEK293T cells. Transfected cells were serum starved and treated with TGF-β in the presence or absence of DHP-2 or SD-208 (Figure 7). Resulting lysates were either treated with alkaline phosphatase or left untreated and resolved on SDS-PAGE gels (Figure 7). In contrast to wild-type ZAK (ZAK-WT), cells overexpressing the catalytically inactive, dominant negative mutant of ZAK (ZAK-dn) lacked higher molecular weight

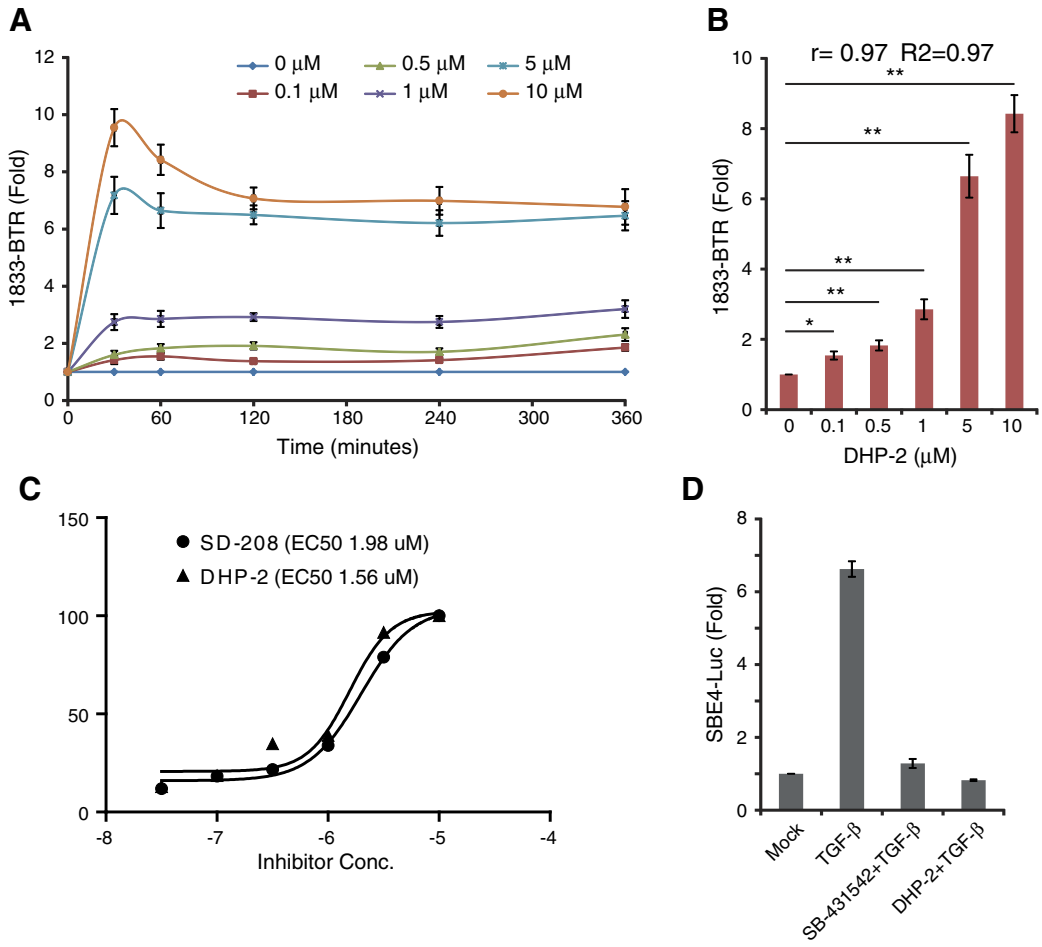

Figure 4. ZAK inhibitor blocks TGF-β signaling in MDA-231-1833 cells. (A) MDA-231-1833 cells stably expressing the BTR reporter were treated with increasing concentrations of DHP-2 (0, 0.1, 0.5, 1, 5 and 10 μM) and bioluminescence activity was measured for up to 6 hours. The change in bioluminescence over mock treatment (DMSO) was calculated and plotted as fold induction. Bioluminescent measurements are in triplicates, error bars denote SEM. (B) BTR reporter fold induction in response to DHP-2 concentrations is plotted to illustrate dose-dependent increase in the reporter activity at 30 minutes post-treatment (maximum reporter activation). Three independent experiments were performed with 8 replicates for each dose. Data points represent the mean ± SEM for each condition. Pearson correlation coefficient (r), goodness of fit (R^2) and statistical significance ($P < .05$ *; $P < .001$ **) is calculated and shown on the plot. (C) 96-Well dose-dependent inhibition of ZAK kinase activity in 1833-BTR cells. Cells treated with ZAK kinase inhibitor (DHP-2) for estimating IC50 as described in Materials and Methods. TGF-β signaling inhibitor SD-208 was used as a positive control for the assay. (D) MDA-231-1833 cells were transfected with SBE4-Luc reporter plasmid and GLuc control plasmid and left untreated or treated with TGF-β (10 ng/mL) or TGF-β in presence of ZAK inhibitor DHP-2 for 24 hours. TGFBR kinase activity inhibitor SB-431542 was used as a positive control. SBE4-Luc bioluminescence activity was measured after addition of luciferin. Values are normalized to Gaussia Luciferase activity and plotted as fold change from mock treated samples. The data shown are representative of at least 3 different biological replicates. Data points represent the mean ± SEM for each condition.

bands representing the autophosphorylated forms of the protein [13]. These higher molecular weight bands were also reduced upon alkaline phosphatase treatment of cell lysates, indicating a role for phosphorylation in their slower migration. Treatment with DHP-2 also resulted in a decrease in these higher molecular weight bands, which could not be further reduced by alkaline phosphatase (Figure 7). In contrast, SD-208 treatment did not significantly impact the levels of these higher molecular weight bands indicating that a TGF-β inhibitor did not significantly alter ZAK kinase activity.

Discussion

Although a role for ZAK in non-canonical TGF-β signaling and hypertrophic growth of cardiomyoblasts through MAPK signaling has been described [10], its role in cancer and TGF-β mediated canonical signaling has not been appreciated. Identification of ZAK in our siRNA screen [4] using a reporter for SMAD-targeted kinase activity (BTR; [3]) was unexpected and prompted us to study this further.

Biochemical studies of the results obtained using the BTR screen confirmed a role for ZAK in TGF-β dependent SMAD2/3 phosphorylation, target gene expression and an invasive cellular phenotype. Since ZAK is a Ser/Thr kinase, we investigated a role for its kinase activity in TGF-β mediated SMAD2/3 signaling using DHP-2, a specific small molecule inhibitor [14]. DHP-2 treatment of BTR expressing A549 lung cancer and MDA-231-1833 breast cancer cells not only activated the BTR reporter in a dose and time dependent manner, but also inhibited SMAD2/3 phosphorylation, target gene expression (SBE4-Luc reporter) and also inhibited cellular phenotypes such as tumor cell invasion. Our finding that DHP-2

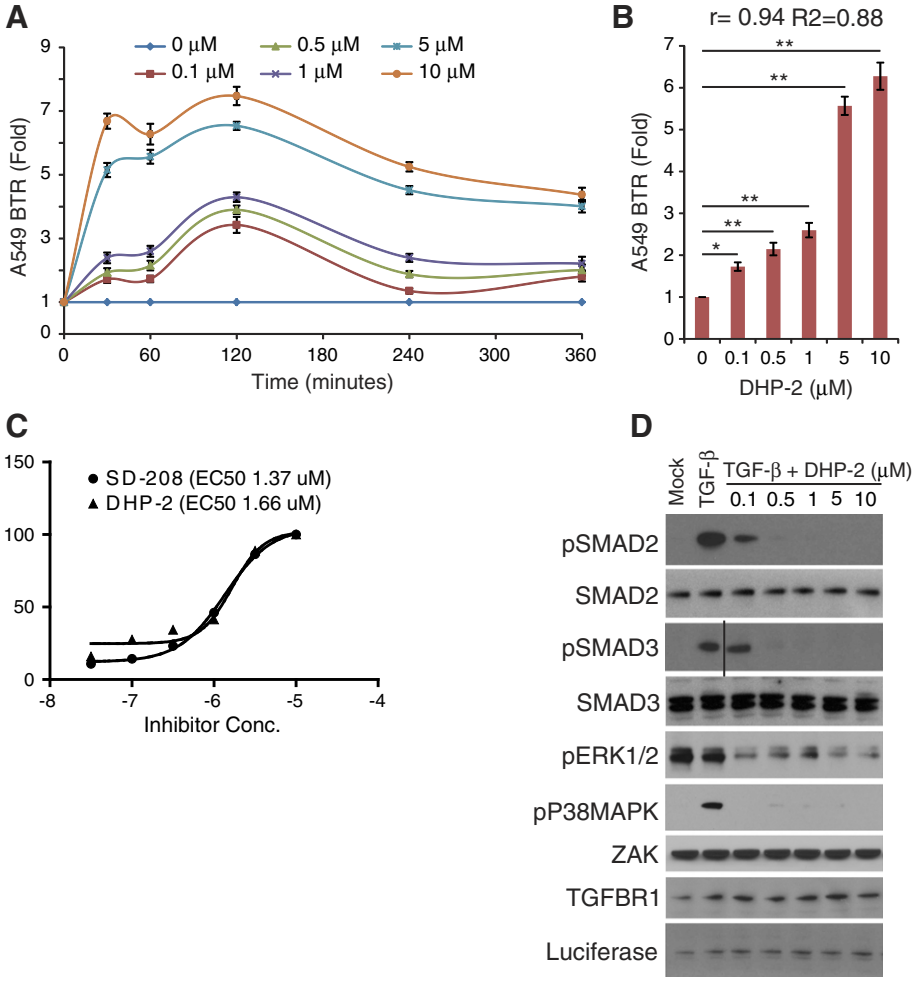

Figure 5. ZAK inhibitor blocks TGF-β signaling in A549 cells. (A) A549-BTR cells were treated with various concentrations of DHP-2 (0, 0.1, 0.5, 1, 5 and 10 μM) and the BTR reporter response was measured serially up to 6 hours. The increase in the bioluminescence activity over DMSO treatment (0 μM) levels was calculated and plotted as fold induction. Bioluminescent measurements are in triplicates, error bars denote SEM. (B) A549-BTR reporter activation in response to DHP-2 concentrations at 30 minutes is plotted to illustrate dose-dependent increase in the reporter activity. Data points represent the mean ± SEM for each condition. Pearson correlation coefficient (r), goodness of fit (R^2) and statistical significance ($P < .05$ *; $P < .001$**) is calculated and shown on the plot. (C) A549-BTR cells were treated with ZAK kinase inhibitor (DHP-2) to estimate the IC50 values in live cells under physiological conditions. Three independent experiments were performed with 8 replicates for each dose. TGF-β signaling inhibitor SD-208 was used as a positive control for the assay. (D) A549-BTR cells were treated with increasing concentrations of DHP-2 (0, 0.1, 0.5, 1, 5 and 10 μM) in presence of TGF-β (10 ng/mL) for 1 hour and resulting lysates were probed with the antibodies indicated. The dividing vertical line in pSMAD3 blot indicate the splice junction.

inhibited TGF-β mediated SMAD2 phosphorylation at sub micromolar concentrations in live cells is consistent with previous results that inhibition of ZAK was also effective at preventing the activation of TGF-β mediated p38MAPK and JNKs in COS-7 and HCT-8 cells [14,21,22] at similar concentrations. Furthermore, as previously reported [10], ZAK was shown to interact with TGFBR1, although inhibition of TGFBR1 kinase activity using SB-431542 in the presence of ligand did not have a significant impact on the interaction of ZAK with TGFBR1 suggesting that ZAK is constitutively bound to the TGFBR1 irrespective of the activation status. A small but reproducible enhancement of the interaction in response to TGFBR1 inhibition was observed. This could be explained by the fact that inhibition of TGFBR1 would lead to decreased internalization and degradation of TGFBR1-TGFBR2 complex [25] and thus leading to the appearance of an enhanced interaction with ZAK.

We also evaluated the possibility that ZAK may interact with SMAD2/3 or SARA. Although as expected, an interaction between SARA and Smad2 was detected in the presence of ligand, an interaction between ZAK and SARA or SMAD2/3 was not detected. In aggregate, although these findings demonstrate a requirement for ZAK and its kinase activity in TGF-β mediated Smad signaling, ligand dependent recruitment of ZAK to the activated receptor or associated accessory proteins was not observed. It is possible that ZAK may interact with TGFBR2 and that this interaction may be conditional on activation, although this possibility was not investigated in our current study.

Although the target substrate of ZAK kinase activity in canonical TGF-β signaling is not yet known, autocatalytic hyperphosphorylation has been demonstrated [10,24], therefore, we evaluated autophosphorylation of ZAK in presence or absence of TGF-β stimulation. Our findings demonstrate that ZAK indeed is

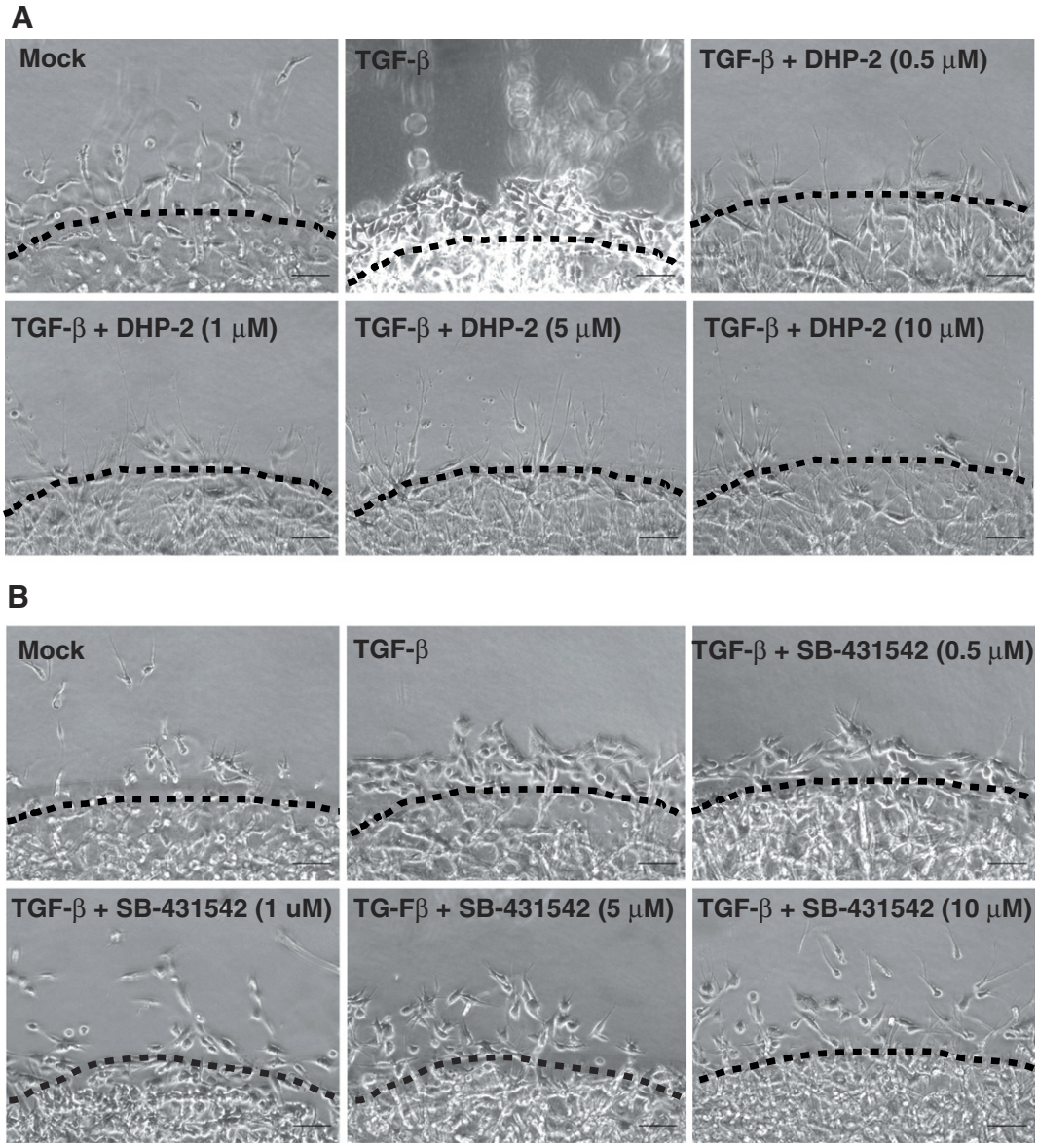

Figure 6. ZAK inhibitor DHP-2 blocks TGF-β mediated cell invasion. MDA-231-1833 cells were plated in hanging drop culture in collagen and allowed to solidify. Media with TGF-β alone (5 ng/mL) or in presence of increasing concentrations of DHP-2 (A) or SB-431542 (B) was added and cells were allowed to invade for 96 hours. Light micrographs were captured and the cell invasion was analyzed by ImageJ.

autophosphorylated since the hyperphosphorylated forms of the protein were not detected when the catalytically inactive mutant (ZAK-dn) was expressed in HEK293T cells. In agreement with the above finding, inhibition of TGF-β signaling using SB-431542 did not reduce hyperphosphorylation of ZAK. A decrease in the slower-migrating (hyperphosphorylated) form of ZAK in the presence of alkaline phosphatase confirmed that this form was due to autophosphorylation. In agreement, the observation that the expression of ZAK-dn did not yield the hyperphosphorylated forms, and in addition treatment with the ZAK inhibitor DHP-2 but not SD-208 (TGFBR1 inhibitor) also resulted in a decrease in the level of the hyperphosphorylated form of the protein, further confirming that the hyperphosphorylation was autocatalytic.

The importance of ZAK and its isoforms in gastric cancer has been emphasized by a recent study [26] wherein more than 50 alternatively spliced forms were shown to be expressed in cancer. The TV1 (ZAK-α) isoform was found to be preferentially up-regulated in gastric, colorectal, bladder and breast cancers tumors and tumor cell lines. Overexpression of the TV1 isoform led to the activation of oncogenic pathways, while siRNA mediated depletion of ZAK in gastric cancer cell lines inhibited their proliferation [26]. Over-expression of ZAK has also been shown to lead to neoplastic cell transformation and tumorigenesis in athymic nude mice [8]. Cell proliferation and neoplastic transformation was abrogated by shRNA mediated depletion of ZAK in epithelial cells [8].

Sorafenib, a multi kinase inhibitor (including C-Raf, B-Raf and VEGF) approved for the treatment of kidney and liver cancers also inhibits ZAK at an IC50 of 48.6 nM [27]. Adverse events of Sorafenib in patients undergoing chemotherapy have been ascribed to the ZAK targeted activity of the agent. Although DHP-2 also inhibits

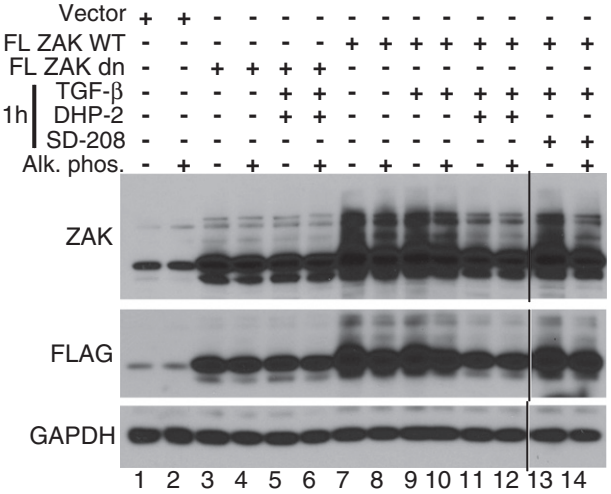

Figure 7. DHP-2 is a ZAK specific inhibitor. (A) HEK293T cells were transfected with plasmids expressing kinase active ZAK (ZAK-WT), ZAK dominant negative (ZAK-dn) or constitutively active ZAK (ZAK-EE), serum starved over-night and treated with TGF-β (10 ng/mL) alone or in presence of SB-431,542 (10 μM) for 1 hour. Resulting lysates were probed with antibodies against ZAK, c-Jun., pERK1/2 and total ERK1/2. (B) HEK293T cells were transfected with ZAK-WT or ZAK-dn, serum starved over-night and treated with TGF-β (10 ng/mL) alone or in presence of SD-208 (10 μM) or DHP-2 (10 μM) for 1 hour. Resulting lysates were incubated with alkaline phosphatase for 1 hour or left untreated and resolved on SDS-PAGE gels and probed with antibodies against ZAK, Flag-tag or GAPDH. The dividing vertical lines in western blots indicate the splice junction.

ZAK at nanomolar concentrations, little information is available on the specificity of DHP-2 on kinases other than ZAKβ, MLK2 and JNK. Our observation that DHP-2 inhibits TGF-β mediated cell migration in MDA-231-1833 cells emphasizes a role for ZAK in TGF-β mediated canonical signaling.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.tranon.2016.09.010.

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