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ZAS3 Accentuates Transforming Growth Factor β Signaling in Epithelial Cells

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Abstract

In mammals, the ZAS family of transcription factors activates or represses transcription depending on the cellular context. In the current study, we explored the interaction between ZAS3 and TGFβ1 signaling in epithelial cells using HEK293 cells and the intestinal epithelial cell line, RIE-1. Endogenous ZAS3 expression was detected in each cell line and the small intestine of mice. Additionally, endogenous ZAS3 expression was increased in both whole cell and nuclear lysates by TGFβ1 and *in vivo* in TGFβ-overexpressing mice, indicating a potential interaction between ZAS3 and TGFβ. ZAS3 transfection enhanced TGFβ1 activation of a luciferase reporter in both HEK293 and RIE-1 cells. Analysis of truncated ZAS3 constructs revealed a 155 amino acid, N-terminal sequence between amino acids 106 and 261 that was required for enhancement of TGFβ1-mediated transcription. Coimmunoprecipitation experiments with nuclear extracts from TGFβ1-stimulated HEK293 cells revealed an association between ZAS3 and the Smad complex. Additionally, transfected ZAS3 decreased the association between the Smad complex and the TGFβ transcriptional repressors Ski and SnoN, indicating a possible mechanism for the enhancement of transcription by exogenous ZAS3. These observations were confirmed by site-directed mutagenesis of ZAS domains homologous with Smad-interacting domains in Ski and SnoN. Finally, ZAS3 transfection enhanced the TGFβ1-mediated induction of α-smooth muscle actin in HEK293 cells, indicating that ZAS3 plays a functional role in TGFβ signaling. In conclusion, we have identified an interaction between ZAS3 and Smad proteins that enhances TGFβ signaling. Since TGFβ signaling is primarily known as a negatively regulated pathway, the enhancement of signaling by ZAS3 has novel implications for understanding TGFβ biology.

Keywords

ZAS3; TGFβ; Ski; SnoN; HEK293

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1. INTRODUCTION

The transforming growth factor beta (TGFβ) family of proteins, including TGFβ, activin, bone morphogenetic proteins (BMPs), and others, regulate diverse cellular functions such as growth, differentiation, adhesion, apoptosis, and migration. Canonical intracellular signaling occurs via the Smad family of signal transducers, including Smad2,3,4,6, and 7. Signaling is initiated when TGFβ ligand engages the TGFβ receptor type II (TGFβRII) dimeric complex. Ligand binding facilitates recruitment and subsequent phosphorylation of the dimeric TGFβ receptor type I (TGFβRI) forming a heterotetrameric active receptor complex [1]. Intracellular signaling is mediated by the Smad proteins, consisting of the R-Smads (Smad2 and Smad3) and Co-Smad (Smad4). The R-Smads are recruited by an activated TGFβ receptor complex and phosphorylated. Phosphorylation of two serine residues at the extreme C-termini of the R-Smads facilitates complex formation with Smad4 [2]. The resulting complex accumulates in the nucleus, binds DNA, and drives transcription of numerous target genes. The Smad DNA binding region was first defined as 5'-GTCTAGAC-3' [3], it was later refined to 5'-GTCT-3' [4] and is referred to as the Smad binding element (SBE). Binding occurs when the zinc containing β-hairpin in Smad3/4 is inserted in the major groove of DNA and is stabilized by hydrogen bonding [4]. Interestingly, the *in vivo* affinity between a single SBE and the Smad complex is too low for binding to occur [4]; although binding can be artificially achieved with concatemers of multiple SBEs, these are rarely found in Smad target promoters and even then do not confer high affinity binding [3,5]. To overcome their deficiency in DNA binding, the Smad proteins rely on multiple cooperating factors to increase DNA binding affinity and supplement transcriptional activity [5]. Examples of common Smad cooperating factors include the chromatin remodeling complex BRG1 [6] and histone modifying enzymes such as the histone acetyltransferases (HATs) p300 and CBP [6,7].

Emerging evidence now indicates a potential role for ZAS proteins in mammalian TGFβ superfamily signaling [8,9]. The ZAS family is comprised of three paralogous proteins in mammals: ZAS1 (also known as HIV-EP1, MBP-1, PRDII-BF1, αA-CRYBP1, and Shn-1), ZAS2 (also known as HIV-EP2, MBP-2, MIBP1, and Shn-2), and ZAS3 (also known as HIV-EP3, KRC, KBP-1, and Shn-3). These proteins are large zinc finger containing transcription factors and have been implicated in growth, signal transduction, immunity, and metastasis (reviewed by Wu [10]). First identified by their ability to bind the NFκB site of various genes, ZAS proteins are large, ~2500 amino acids that share a distant relation to *Drosophila* Schnurri (Shn) [11,12] Maekawa *et al*. [13]) (reviewed by Raftery and Sutherland [14]).

Observations in ZAS2$^{-/-}$ mice and mouse embryonic fibroblasts (MEFs) indicate a role for ZAS2 in adipogenesis [8]. ZAS2$^{-/-}$ mice have decreased white adipose tissue and furthermore, ZAS2$^{-/-}$ MEFs were unable to differentiate into adipocytes *in vitro*. It was determined that ZAS2 acts as a critical regulator in BMP signaling and adipocyte differentiation. Following stimulation by BMP-2, ZAS2 interacts with Smad1/4 and C/EBPα in the nucleus to drive expression of PPARγ2. PPARγ, a member of the PPAR family of transcriptional factors, drives expression of several adipocyte-specific genes and induces adipocyte differentiation [15,16]. Furthermore, *Drosophila* Shn1 has been shown to drive expression of a *Xenopus* BMP response element (BRE) [9], further implicating Shn/ZAS as modulators of TGFβ signaling.

Based on the aforementioned roles of the Shn/ZAS proteins in Dpp and BMP signaling, we sought to determine if ZAS3 plays a role in the canonical TGFβ signaling pathway mammalian epithelial cells. Herein we show ZAS3 accentuates Smad-dependent TGFβ signaling in the epithelial cell lines HEK293 and RIE, and propose a novel mechanism where ZAS3 negatively regulates Ski/SnoN function by competing for binding to Smad proteins.

2. EXPERIMENTAL PROCEEDURES

2.1 Cell culture and tissue isolation

HEK293, RIE, RIE-Ras, and Caco-2 cells were obtained from American Type Culture Collection (ATCC). Cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (HEK293, RIE-1) and 20% (Caco-2) fetal bovine serum (FBS). Cells were cultured at 37 °C in a humidified environment containing 5 % CO_2. Small intestine segments were isolated from 10-week old wild-type (w.t.) FVB/N mice or transgenic mice, designated vil-TGFβ1, which overexpress constitutively active TGFβ1 in the intestine under the control of a villin promoter (J.A. Barnard, unpublished data). Lysates prepared from the small intestine were homogenized and subjected to Western blotting with the anti-ZAS3 antibody described below.

2.2 Generation of full length and truncated ZAS3 constructs

ZAS3 expression constructs were synthesized as previously described [17].

2.3 Cell transfection, stimulation, and luciferase assays

Cells were plated in 24-well plates (Costar) at a density of 3×10^5 cells/well in a final volume of 0.5 mL and allowed to adhere for 24 h. The medium was removed and replaced with 0.5 mL/well of transfection medium consisting of 75% opti-MEM (Invitrogen, Carlsbad, CA) and 25% DMEM supplemented with 10% FBS. Cells were transfected with 0.8 μg DNA/well consisting of 0.3 μg TGFβ-inducible reporter construct 4×-SBE-luc [5] (kindly provided by Dr. Joan Massague), 0.01 μg of pRL-SV40 (Promega), and 0.5 μg of the indicated ZAS3 plasmid construct or control vector. The plasmids were combined with transfection reagent containing 2.4 μL PEI [18] (Polysciences, Warrington, PA) in 20 μL Opti-MEM per well and incubated at room temperature for 20 min. Following incubation, the plasmids were added dropwise to wells and plates were incubated for 24 h. The medium was removed and replaced with 0.5 mL DMEM (10% FBS). Cells were stimulated by 24 h incubation with either porcine TGFβ1 (2 ng/mL) (R&D Systems, Minneapolis, MN) or vehicle control. After stimulation, cell extracts were prepared and luciferase assays were performed using the Dual-luciferase reporter assay system (Promega, Madison, WI) and analyzed using a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA). Transfection efficiencies were normalized based on *Renilla* luciferase activity. Experiments were performed at least three times and representative results are shown.

For Western blotting and co-immunoprecipitation (co-IP) assays, cells were plated at a density of 10^6 cells/150 mm petri dish (Costar) in 25 mL DMEM (10% FBS). Transfections were performed as described above with the exception that cells were transfected solely with the indicated construct (20 μg/dish) using 3 μL PEI/μg DNA in Opti-MEM. Twenty four hours post-transfection, medium was replaced with DMEM (10% FBS) and cells were stimulated with TGFβ1 (2 ng/mL) or vehicle control for 1 h unless indicated otherwise. After stimulation, cells were harvested for Western blotting or purification of nuclear proteins as described subsequently.

2.4 Isolation of whole cell and nuclear proteins

To isolate soluble cellular proteins, cells were washed twice with cold PBS at 4 °C then lysed with 100 μl of RIPA lysis buffer (50 mM Tris (pH7.4), 1% NP-40, 0.25% deoxycholate, 50 mM NaCl, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, and protease complete inhibitor cocktail (Roche Diagnostics, Indianapolis, IN)). Insoluble cell particulate was removed by centrifugation at 16000 × g. To prepare nuclear extracts, cells were washed 3× with chilled phosphate buffered saline (PBS). After washing, 0.8 mL of isotonic buffer (0.14 M NaCl, 1.5

mM $MgCl_2$, 10 mM Tris, 0.5% NP40, 20 mM β-glycerophosphate, complete protease inhibitor cocktail (Roche Diagnostics)) was added to each well. Cells were scraped from plates with a rubber policeman, transferred to pre-chilled 1.5 mL Eppendorf tubes, and incubated on ice for 30 min. Nuclear material was pelleted by centrifugation at 14,000 × *g* for 10 min. The cytosol-containing supernatants were discarded and the nuclear pellets were resuspended in 600 μL of chilled extraction buffer (150 mM NaCl, 50 mM Tris (pH 7.4), 1% NP40, 0.25% SDS, complete protease inhibitor cocktail (Roche Diagnostics), 20 mM β-glycerophosphate, 10% glycerol) and gently rocked at 4 °C for 15 min. Samples were then vortexed for 30 s and gently rocked at 4 °C for an additional 15 min. Insoluble particulate was removed by centrifugation at 14,000 × *g* for 6 min, the nuclear protein containing supernatants were removed and transferred to pre-chilled tubes. Protein concentrations of whole cell and nuclear extracts were determined by the BCA protein assay (Thermo Scientific, Waltham, MA) and absorbencies were measured on a SpectraMax M2 (Molecular Devices, Silicon Valley, CA).

2.5 Antibodies

Antibodies used were: mouse monoclonal anti-α-smooth muscle actin (SMA) (Sigma), rabbit polyclonal anti-pSmad2 (Ser465/467, Cell Signaling Technology, Danvers, MA), goat polyclonal anti-Smad2 (Santa Cruz Biotechnology, Santa Cruz, CA), mouse monoclonal anti-Smad4 (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-cSki (Prosci, Poway, CA), rabbit polyclonal anti-SnoN (Abcam, Cambridge, MA), and mouse monoclonal anti-tubulin (Santa Cruz). The anti-ZAS3 antibody has been described previously [19]. All primary antibodies were diluted 1:2000 in blocking buffer with the exception of anti-tubulin and anti-Smad4 antibodies, which were diluted 1:1000. Secondary IgG antibodies were diluted 1:10,000 in blocking buffer.

2.6 Immunoprecipitation and immunoblotting

For immunoprecipitation, 200 μg of cellular or nuclear proteins were combined with 2 μL or 3 μL of rabbit anti-pSmad2 or mouse anti-Smad4 antibodies, respectively, and gently rocked overnight at 4 °C. Samples were then clarified by centrifugation at 14,000 × *g* for 5 min. Supernatants were transferred to chilled tubes and combined with 30 μL of a 50% slurry of protein G beads (Millipore, Billerca, MA) per sample. The samples were incubated at room temperature for 30 min, and then centrifuged at 400 × *g* for 3 min. The supernatants were discarded and the pellets were washed 3× with 1 mL of chilled wash buffer. Immunoprecipitates and soluble cellular proteins were resolved by SDS-PAGE and transferred to PVDF membranes in transfer buffer (25 mM Tris, 192 mM glycine, and 20% methanol) at 20 V for 50 min in a Trans-Blot SD Semi-dry transfer cell (BioRad, Berkley, CA). Membranes were then blocked, incubated with the designated primary antibody overnight at 4 °C, washed, and incubated with horseradish peroxidase-conjugated IgG for 1 h. The Enhanced Chemiluminescence Plus Detection System (GE Healthcare, Piscataway, NJ) was used to detect the antigen-antibody complexes. Densitometry measurements were performed using a FluoroChem 8900 (Cell Biosciences, Salt Lake, UT).

2.6 Sequence homology searching and establishment of ZAS3(T1) mutant constructs

Sequence homology searching was performed using ClustalW [20] to align the amino acid sequences of cSki (PubMed ID# P12755) and SnoN (PubMed ID# AAH59386) with ZAS3 (T1). Homologous regions were identified in ZAS3 and mutated to alanine using the QuickChange® Site-Directed Mutagenesis Kit (Agilent Technologies). The primer sequences used for mutagenesis were as follows: M(SLQH) 5'-GAGCAATCACTGCTGGCCGCGGCCGCCCCACCCAGCTCCACGCAC-3' plus reverse complement, M(SLKP) 5'-CGTCCCTCACAGGTCGCTGCAGCTGCTGCAGAAGAGGCACACAAG-3' plus reverse

complement, and M(SLQP) 5'-GGACGCTCCGCTCACGCTGCAGCTGCTAGGTTGGTCCGGCAGCCC-3' plus reverse complement. Mutations were confirmed by restriction digest of newly introduced NotI, in M (SLQH), and PstI, in M(SLKP) and M(SLQP), restriction enzyme sites and subsequently by sequencing (DNA sequencing core, The Research Institute at Nationwide Children's Hospital). For each immunoprecipitation, experiments were performed at least three times. Representative Western blots are shown.

3. RESULTS

3.1 ZAS3 is induced by TGFβ1 and accentuates Smad signaling

We first determined if TGFβ1 signaling elicited an effect on endogenous ZAS3 levels to explore potential interactions between TGFβ1 and ZAS3. Treatment of HEK293, RIE, and RIE-Ras cells with exogenous TGFβ1 (2 ng/mL) induced a time dependent increase in endogenous ZAS3 levels, with maximum induction occurring 24-hours post-treatment (Figure 1A). Dramatic increases of endogenous ZAS3 levels were also detected in the small intestine of vil-TGFβ1 transgenic mice (Figure 1B). The vil-TGFβ1 mouse overexpresses constitutively active TGFβ1 specifically in the small intestine under the control of a villin promoter (unpublished data). These findings are significant as they illustrate an *in vivo* relationship between TGFβ1 and ZAS3.

To investigate if ZAS3 modulates TGFβ signaling, HEK293, RIE-1, and Caco-2 cells were transiently co-transfected with full-length (FL) ZAS3 and the 4×SBE-Luc reporter construct. Stimulation of transfected HEK293 and RIE-1 cells with TGFβ1 showed a marked accentuation of luciferase activity, ~3-fold over control vector transfected cells in the HEK293 line (Figure 2A). These results were confirmed using an alternate Smad activated reporter assay, the 3TP-luciferase assay (data not shown). To confirm ZAS3 expression levels, Western Blotting was performed using whole cell lysates from ZAS3 and control vector transfected HEK293 cells (Figure 2B). Moreover, a dose-dependent relationship is observed between TGFβ signal accentuation and the amount of ZAS3(FL) transfected in both HEK293 and RIE-1 cell lines (Figures 2C and 2D, respectively). To investigate if the ZAS3-mediated signal accentuation was Smad-dependent, Caco-2 cells, which have inactivating mutations in Smad4 but functional Smad2/3 [21], were transfected with ZAS3. Stimulation of transfected Caco-2 cells with TGFβ1 did not activate the luciferase reporter (Figure 2A) indicating signal accentuation is dependent on the assembly of a fully functional Smad complex. These observations led us to further examine the mechanism by which ZAS3 accentuates TGFβ signaling.

3.2 Structure activity relationship and mechanistic analysis of ZAS3-mediated TGFβ signaling accentuation

To identify structural elements in ZAS3 required for TGFβ signal accentuation, a series of truncated constructs were synthesized and co-transfected along with the 4×SBE-luc reporter into HEK293 cells (Figure 3A). Transfected cells were then stimulated with TGFβ1 (Figure 3B). The largest TGFβ signal accentuation was obtained by construct ZAS3(T1), which retained all three zinc finger domains, but was C- and N-terminus truncated. Comparison of constructs ZAS3(T1) and ZAS3(T3) indicated an important role for the double zinc-finger region directly preceding amino acid 261 in accentuation of TGFβ1 signaling. Furthermore, the decreased activity of ZAS3(T3) indicated the presence of a functionally relevant motif located between a.a.750 and 1186. The C-terminus of ZAS3 appears to be functionally insignificant for accentuation of TGFβ signaling.

To investigate the underlying mechanisms of ZAS3-mediated TGFβ signal accentuation, immunoblotting (IB) and immunoprecipitation (IP)/IB experiments were performed using

nuclear extracts from TGFβ1-stimulated HEK293 cells transfected with ZAS3 constructs. To confirm preliminary data indicating that ZAS3 accentuates TGFβ signaling through the Smad pathway, we probed for nuclear accumulation of pSmad2 and pSmad2/Smad4 heteromeric complexes in the presence and absence of ZAS3 (Figure 3C). TGFβ1-stimulated HEK293 nuclear extracts exhibited the greatest accumulation of pSmad2 when the cells were transfected with ZAS3(T1), the most active construct, as shown in the top panel. In contrast, the ZAS3 (T4) construct, which failed to stimulate 4×-SBE luciferase activity (Figure 3B), was ineffective in stimulating pSmad2 nuclear localization. Nuclear Smad4/pSmad2 complex levels were also increased by transfection with ZAS3(T1). As the N-term Zn-finger regions in ZAS3 were necessary to enhance TGFβ signaling, and could potentially interact at or near Smad-DNA interface, we searched for potential associations between ZAS3 and the Smad complex. Using nuclear extracts from HEK293 cells, we show that ZAS3 co-immunoprecipitates with pSmad2 (Figure 3C). Taken together, these data implicate ZAS3 as a novel enhancer of canonical TGFβ signaling that associates with the DNA-binding Smad complex.

Notwithstanding the apparent simplicity of canonical TGFβ signaling, the presence of multiple co-repressors and co-activators adds a level of complexity and versatility to Smad dependent signaling (reviewed by Massague et al. [22]). Negative regulation of canonical TGFβ signaling occurs at virtually every level from receptor to transcriptional activation (reviewed by Itoh and ten Dijke [23]). As such, to examine the potential underlying mechanisms of ZAS3-mediated TGFβ signal accentuation, we investigated potential interactions between ZAS3 and negative regulators of TGFβ signaling. Given our experimental evidence of a nuclear ZAS3/Smad complex, we first examined known negative regulators of TGFβ signaling that act within the nucleus. Currently, there are several known mechanisms of negative regulation that occur in the nucleus, including: 1) sequestration of the Smad2/3 complex to the inner nuclear membrane by the integral nuclear membrane protein Man1; 2) dephosphorylation of the pSmad2 by PDP, PPM1A, and SCP1, 2, and 3; 3) interference of the interactions between the Smad complex and additional transcription factors by LIP; and 4) disruption of the Smad2/3-Smad4 complex and/or interference of association with co-activators and recruitment of HDAC by Ski, SnoN, or TGIF [23]. We found the TGFβ1 antagonists Ski and SnoN of particular interest with respect to ZAS3 function since these co-repressors each have a C_2H_2 Zn-binding module (reviewed by Deheuninck and Luo [24]). Ski and SnoN belong to the Ski family of nuclear protooncogenes and exert TGFβ antagonistic effects by interacting with Smad proteins, disrupting complex formation, preventing binding of co-activators, and recruiting co-repressors [25–31].

Co-IP experiments were performed to search for a potential interaction between ZAS3 and Ski/SnoN in HEK293 cells. Interestingly, when cells were transfected with ZAS3(T1) and treated with TGFβ1 there was a marked reduction in the association between both Ski and SnoN with pSmad2 in nuclear extracts (Figure 3C). No alterations in the Ski/SnoN-pSmad2 complex were observed with cells are transfected with the control vector or the inactive ZAS3(T4) construct. These results indicate that ZAS3(T1) attenuated the association between pSmad2 and cSki/SnoN and offer insight into a potential mechanism underlying the observed TGFβ signal accentuation by ZAS3. To further investigate and validate the apparent relationship between ZAS3 and Ski/SnoN, cells were co-transfected with ZAS3(T1) and SnoN to look for mitigating effects on TGFβ signal accentuation (Figure 3D). Cells transfected with SnoN showed a decrease in luciferase activity, while transfection with ZAS3(T1) alone resulted in the expected dramatic increase. When cells are co-transfected with ZAS3(T1) and SnoN, luciferase activity was significantly attenuated when compared with ZAS3(T1) transfectants alone. The observed mitigation of signal accentuation supports the aforementioned observation that ZAS3 attenuated the interaction between Ski/SnoN and the active Smad complex. To mechanistically confirm the 4×-SBE-Luc results with co-transfected cells, IP/IB was performed with the

HEK293 nuclear extracts (Figure 3E). As we previously observed, transfection with ZAS3(T1) decreased the association between pSmad2 and endogenous cSki/SnoN following TGFβ1 stimulation. ZAS3(T1) transfection also increased nuclear Smad4/pSmad2 complex levels and pSmad2 levels. Co-transfection with SnoN partially restored cSki/SnoN-pSmad2 complex levels and decreased Smad4-pSmad2 and pSmad2 levels when compared to ZAS3(T1) transfectants alone. These data further indicate that ZAS3's regulatory potential is derived from antagonism of Ski/SnoN.

3.3 Site directed mutations add additional evidence that ZAS3 accentuates TGFβ signaling by antagonizing Ski/SnoN

To further define the modulatory role ZAS3 plays in Ski/SnoN function, we sought to clarify the molecular mechanisms of inhibition. Previously, regions in Ski/SnoN that interact with the Smad proteins have been identified [32]. Ski/SnoN interacts with both R-Smad proteins (Smad2 and Smad3) and Smad4, albeit via different motifs. Major and minor Ski/SnoN binding regions have been identified within Smad2/3. The minor binding site is located between residues 241–323; however, it is not absolutely required for interaction with Smad2/3 or signal repression [26,32]. The major binding site occurs at to two amino acid motifs, GLQH and SLKH, in Ski and SnoN, respectively. Mutations at these sites abolished interactions with the R-Smads, but residual interaction with Smad4 appears to be sufficient for signal repression. Multiple amino acid residues, including His^{222}, Glu^{223}, and Trp^{274} in Ski and the conserved His^{266}, Glu^{267}, and Trp^{318} in SnoN are found to be required for association with Smad4.

A homology search of ZAS3(T1) using ClustalW [20] identified three regions that share close homology with the GLQH and SLKH residues that mediate Ski/SnoN-R-Smad interactions. The residues $SLKP^{88–91}$, $SLQH^{440–443}$, and $SLQP^{747–750}$ were identified as highly homologous to the Ski/SnoN GLQH and SLKH residues. To assess if the homologous residues identified in ZAS3 conveyed functional significance, site-directed mutagenesis was used to mutate the indicated residues in ZAS3(T1) to alanine. The mutated constructs were designated M(SLKP), M(SLQH), and M(SLQP) (Figure 4A). 4×-SBE-luc assays performed on cells transfected with the three mutant constructs (Figure 4B) showed a decrease in ZAS3-mediated TGFβ signal accentuation. As mutation of the SLQH motif was the most deleterious to the signal accentuating activity of ZAS3, M(SLQH) was chosen for further evaluation. To mechanistically evaluate M(SLQH), nuclear extracts from stimulated HEK293 cells transfected with ZAS3(T1), M(SLQH), or control vector were analyzed by IP/IB (Figure 4C). As expected, ZAS3(T1) increased levels of Smad4-pSmad2 complex and decreased levels of association between pSmad2 and cSki/SnoN when compared to control vector. In contrast, M (SLQH) failed to increase Smad4-pSmad2 levels and more notably, failed to dissociate cSki and SnoN from pSmad2. These results, taken together with 4×SBE-luc data, indicate a mechanism where ZAS3 accentuates TGFβ signaling by directly competing with cSki/SnoN for binding to the R-Smad complex.

3.4 Transfection with ZAS3(T1) increases α-SMA levels in HEK293 cells

Finally, to investigate the biological consequences of ZAS3-mediated TGFβ1 signal accentuation, we examined α-SMA levels in transfected HEK293 cells. TGFβ1 is a well known inducer of α-SMA production in various cell types [33–36], and α-SMA is markedly increased in the small intestine of vil-TGFβ1 mice (unpublished observations). Transfection of HEK293 cells with ZAS3(T1) and stimulation with TGFβ1 induced modest increases in α-SMA levels when compared to control vector (Figure 4C). Furthermore, densitometry analysis of ZAS3 (T1) transfected cells indicated TGFβ1 stimulation increased α-SMA levels by 48% when compared to control vector. This phenotypical observation is a hallmark of accentuated TGFβ1 signaling and demonstrates that ZAS3 has functional significance within the canonical TGFβ signaling pathway. To confirm previous observations indicating a functional

significance of the SLQH motif, HEK293 cells were transfected with M(SLQH) (Figure 4C). Densitometry analysis comparing ZAS3(T1) and M(SLQH) transfected cells indicates that transfection with M(SLQH) reduces TGFβ1 mediated α-SMA induction by 15%; this is in agreement with our previous findings demonstrating decreases in 4×-SBE-luc activity and reduced affinity between ZAS3 and the Smad complex following mutation of the SLQH residues.

4. DISCUSSION

We investigated ZAS3 as a modulator of TGFβ signaling based on its phylogenetic relationship to drosophila Shn, a known regulator of dpp, which is homologous to TGFβ in mammals. Shn directly binds to the pMAD-Medea complex, homologous to mammalian pSmad2/3-Smad4 complex, and represses transcription of Brk, a negative regulator of dpp signaling (reviewed by Affolter and Balser [13]). Although Brk is not found outside of anthropods, the Shn-related ZAS proteins regulate Bmp signaling, a member of the TGFβ superfamily of proteins [8,9]. Previously, ZAS1 and ZAS2 have been shown to modulate Bmp signaling by interacting with Smad1/4 and subsequently recruiting/interacting with additional co-activators or co-repressors to mediate transcription [8,9]. Potential interactions between ZAS3 and Bmp-related Smads have not been investigated. We selected ZAS3 for study in the current work based on our prior observations that disrupted ZAS3 expression in mammalian cell lines resulted in accelerated cell proliferation, and that tumors have been observed in ZAS3 deficient mice, indicating a potential role for ZAS3 in modulating cell growth and proliferation [37,38]. More recently, we reported that $ZAS3^{-/-}$ mice have alterations in T-cell development and activation, and an increase in bone density [11]. Taken together, the effects of the Zas family on growth regulation, tumor inhibition, and T-cell development, all of which are cellular processes regulated by TGFβ, we chose to investigate a potential role for ZAS3 in mammalian TGFβ signaling.

As multiple regulators of TGFβ signaling, both positive and negative, are either induced or repressed in response to TGFβ (reviewed by Massague and Miyazono [39,40]), the observed induction of endogenous ZAS3 in TGFβ1-treated HEK293 cells, RIE-1 cells, and in the small intestine of TGFβ1 overexpressing transgenic mice indicated a potential regulatory role for ZAS3 in the TGFβ signaling pathway. Indeed, it was determined that ZAS3 accentuates Smad-dependent TGFβ signaling assessed by 4×-SBE-luc luciferase and 3TP-luciferase assays. In attempt to determine the structure activity relationship of ZAS3-mediated signal accentuation, several truncated constructs were synthesized. The structural insight gathered from this approach indicated the necessity for the N-term Zn-finger regions and subsequent amino acid residues extending to a.a. 1186. As the greatest signal enhancement was observed with truncated construct ZAS3(T1), the C-terminus of ZAS3 appears functionally insignificant for TGFβ signal modulation.

Previous observations that ZAS1 and ZAS2 mediate BMP signaling by binding to Smad1 and Smad4 [8,9], suggest to us experiments investigating interactions between the TGFβ associated Smads and ZAS3. ZAS3(T1) increased nuclear pSmad2 and pSmad2-Smad4 levels and coimmunoprecipitated with pSmad2. It should be noted that the co-IP experiments performed herein are limited in that they cannot conclusively determine if ZAS3 interacts individually with Smad2, Smad3, or Smad4, as the Smads immunoprecipitate as a complex. A drawback of the current work is the inability to study the inverse relationship (i.e. TGFβ1 signaling in the absence of ZAS3) between TGFβ signaling and ZAS3 levels. We unsuccessfully attempted use of multiple siRNAs to inhibit ZAS1, ZAS2 and ZAS3 signaling, but were unsuccessful, presumably due to redundancy in the ZAS protein family.

As there are a multitude of Smad interacting proteins (reviewed by Brown *et al.* [41]), we chose to examine those that negatively regulate TGFβ signaling in an attempt to unravel the mechanism by which ZAS3 accentuates TGFβ signal transduction. Although extensive negative regulation of canonical TGFβ signaling can occur at many levels [23], we focused on the well-studied Ski family of oncoproteins as potentially intriguing candidates.

Ski was first identified based on its ability to transform chicken embryo fibroblasts and was named based on its viral homologue, v-Ski, the transforming component of the Sloan-Kettering virus (SKV) [27,42]. Subsequently, SnoN (Ski related novel protein) was identified based on its homology to v- and c-Ski [27]. The primary function of Ski and SnoN is transcriptional regulation, which occurs by interaction with numerous other transcriptional modulators [24]. Ski and SnoN exert their TGFβ antagonistic activities primarily by disruption of the active complex by simultaneous binding to Smad2/3, via their N-term region, and Smad4, via a C_2H_2 Zn-binding module [31]. Furthermore, the inactive Smad-Ski/SnoN complex may further attenuate TGFβ signaling by: 1) competing with the active Smad complex for DNA binding [43]; 2) preventing binding of the transcriptional co-activator p300/CBP [31]; and 3) recruiting a transcriptional co-repressor complex containing N-CoR and HDAC [26,30]. As Ski and SnoN act in the nucleus, bind the Smad complex, and contain Zn-binding module they were identified as potential ZAS3 modulators. Indeed, the ability of ZAS3(T1) to displace Ski and SnoN from pSmad2, and presumably the entire Smad complex, indicated a potential interrelationship among these proteins.

Further examination of the relationship between Ski/SnoN and ZAS3 by site directed mutagenesis revealed a potential mechanism where ZAS3 accentuates TGFβ signaling by directly competing with Ski/SnoN for binding to the Smad complex, more specifically, Smad2 and Smad3. It is not clear at what point during TGFβ signal transduction the competition between ZAS3 and Ski/SnoN occurs, as Ski/SnoN have been shown to interact with the Smad proteins at various stages during complex formation [31,44,45]. It is also not yet clear which Smad proteins directly interact with ZAS3. The decrease in ZAS3 activity and Smad affinity following mutation of residues that share homology to the reported Smad2/3 binding domain of Ski/SnoN [32] indicate that ZAS3-Smad2/3 association is likely. However, the ability of Ski/SnoN to repress TGFβ signaling independent of interactions with Smad2/3 [32] brings into question if ZAS3 competition for Smad2/3 binding alone is sufficient to antagonize Ski/SnoN-mediated signal repression. The ability to co-IP ZAS3 and Smad4 is indicative of a potential additional interaction, however, the possibility that Smad4 is precipitated due to its interactions with Smad2/3 cannot be excluded and more detailed study is needed. The N-term Zn-finger motifs in ZAS3 are of particular interest in this regard. Based on the data presented herein, two potential roles can be envisioned for the ZAS3 Zn-finger domain. ZAS3 could bind DNA to enhance affinity/transcription at the Smad binding region or compete with Ski/SnoN for binding to Smad4. The ability of ZAS3 to displace Ski/SnoN from Smad2/3 and, presumably, the Smad complex supports the latter hypothesis; that is there is competition at the Smad4 binding site, however, due to the large size of ZAS3, it is possible that interactions with Smad2/3 sterically inhibit interactions between Smad4 and Ski/SnoN.

5. CONCLUSIONS

Collectively, our results implicate ZAS3 as a novel modulator of canonical TGFβ signaling. We propose a model where ZAS3 competes with the TGFβ repressors Ski and SnoN to accentuate TGFβ signaling (Figure 5). As it has been suggested that other cofactors, including SKIP [46] and CBP [47], also compete at the Ski/SnoN-Smad binding site, comprehensive efforts directed towards investigating the interactions between the multiple cofactors, placed within stoichiometric context, and the Smads are paramount. Furthermore, *in vivo* studies utilizing a ZAS3 deficient mouse model [11] along with mice that over- or under-express

TGFβ will prove useful in further characterizing the biological relevance of ZAS3-mediated TGFβ signal accentuation.

Acknowledgments

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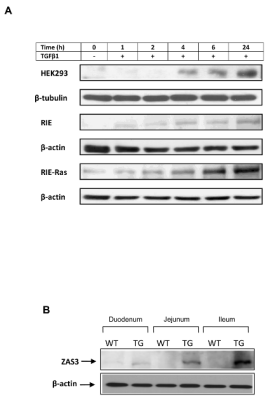

Fig. 1. TGFβ1 induces ZAS3 expression *in vitro* and *in vivo*

A, ZAS3 expression levels were assessed in HEK293 cell lysates at the indicated time points following stimulation with porcine TGFβ1 (2 ng/mL). Induction of ZAS3 was first observed 4 h post-stimulation and peaked at 24 h. B, ZAS3 levels in the small intestine of wild-type and vil-TGFβ1 mice. Overexpression of constitutively active TGFβ1 in the small intestine induces a marked increase in ZAS3 levels. Results shown are representative of multiple independent experiments

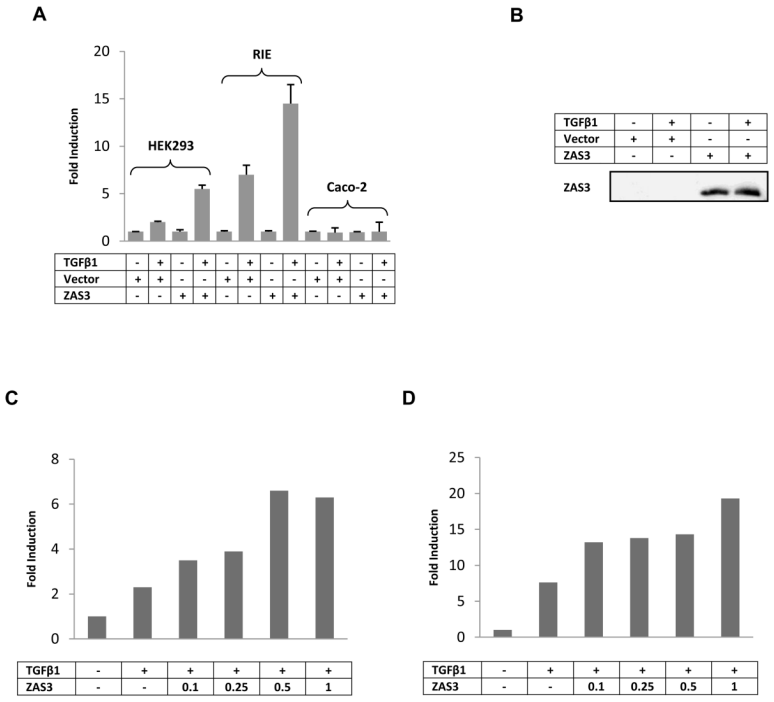

Fig. 2. Transfection with ZAS3 increases 4×SBE-Luc activity in HEK293 and RIE-1 cells

A, HEK293 cells were co-transfected with ZAS3 (0.5 μg/well) or control vector and the 4×SBE-Luc reporter construct (0.3 μg/well). Medium was replaced 24 h post-transfection with DMEM (10% FBS) and cells were stimulated with porcine TGFβ1 (2 ng/mL) for 24 h prior to measurement of luciferase activity. Values are means from multiple independent experiments ± S.D. B and C, Transfection of HEK293 and RIE-1 cells, respectively, with increasing concentrations of ZAS3 (μg/well) shows a dose-response relationship. Maximum luciferase intensity levels are achieved by transfection with 0.5 μg/well and 1 μg/well in HEK293 and RIE-1 cells, respectively.

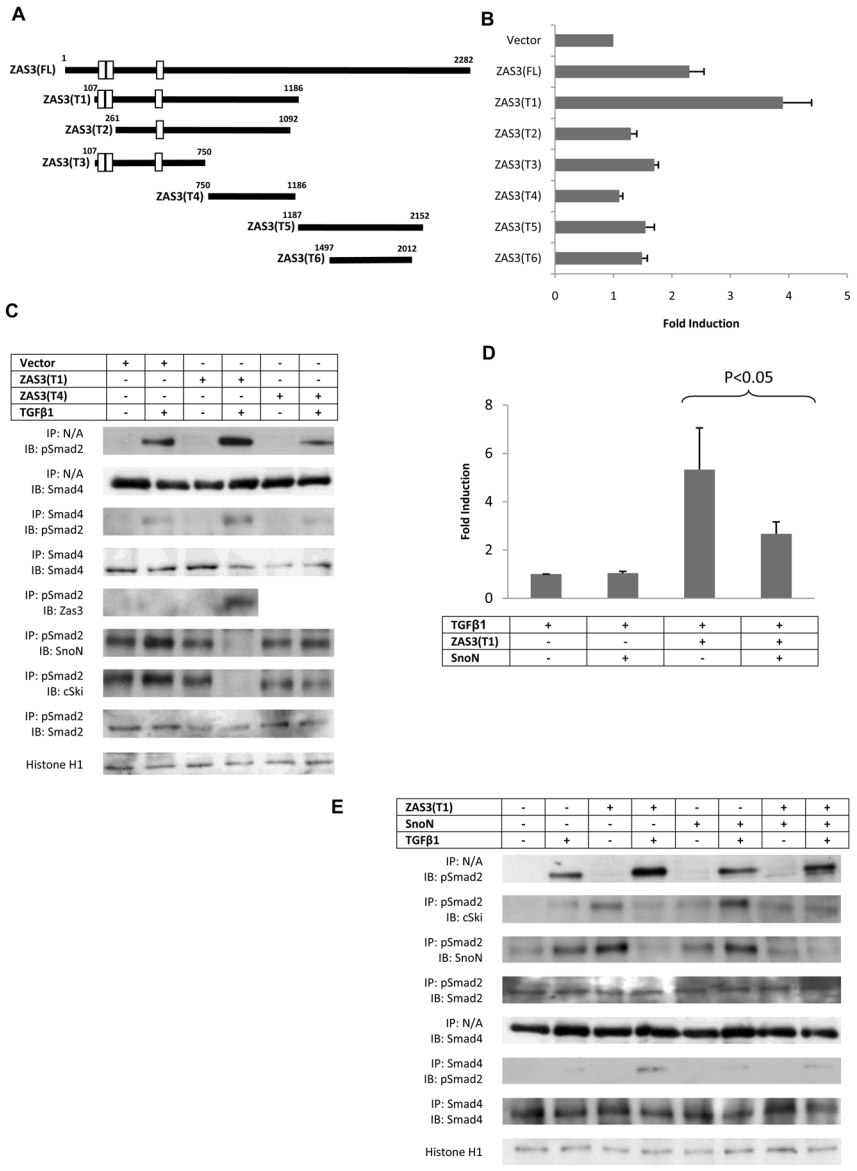

Fig. 3. Truncated ZAS3 constructs give insight into functional attributes of ZAS3 and reveal a potential mechanism behind TGFβ1 signal accentuation

A, Full length (FL) and truncated (T) ZAS3 constructs were created, single- (▯), and double-zinc finger (▯▯) regions are indicated. B, HEK293 cells were co-transfected with indicated constructs (0.5 μg/well) and 4×SBE-Luc reporter (0.3 μg/well). Medium was replaced 24 h post-transfection with DMEM (10% FBS) and cells were stimulated with TGFβ1 (2 ng/mL) for 24 h prior to measurement of luciferase activity. C, Physical interactions between Smad proteins and ZAS3, SnoN, and Ski in nuclear extracts from ZAS3 transfected (0.5 μg/well), TGFβ1 (2 ng/mL) stimulated (1 h), HEK293 cells. D, HEK293 cells were co-transfected with ZAS3(T1) (0.5 μg/well), SnoN (0.5 μg/well), and 4×SBE-Luc (0.3 μg/well). Medium was replaced 24 h post-transfection with DMEM (10% FBS) and cells were stimulated with TGFβ1 (2 ng/mL) for 24 h prior to measurement of luciferase activity. E, Physical interactions between Smad proteins and Ski/SnoN in nuclear extracts from ZAS3 (0.5 μg/well) and SnoN (0.5 μg/well), TGFβ1 (2 ng/mL) stimulated (1 h), HEK293 cells. Luciferase values are means

from at least three independent experiments ± S.D., co-IP results are representative of multiple independent experiments.

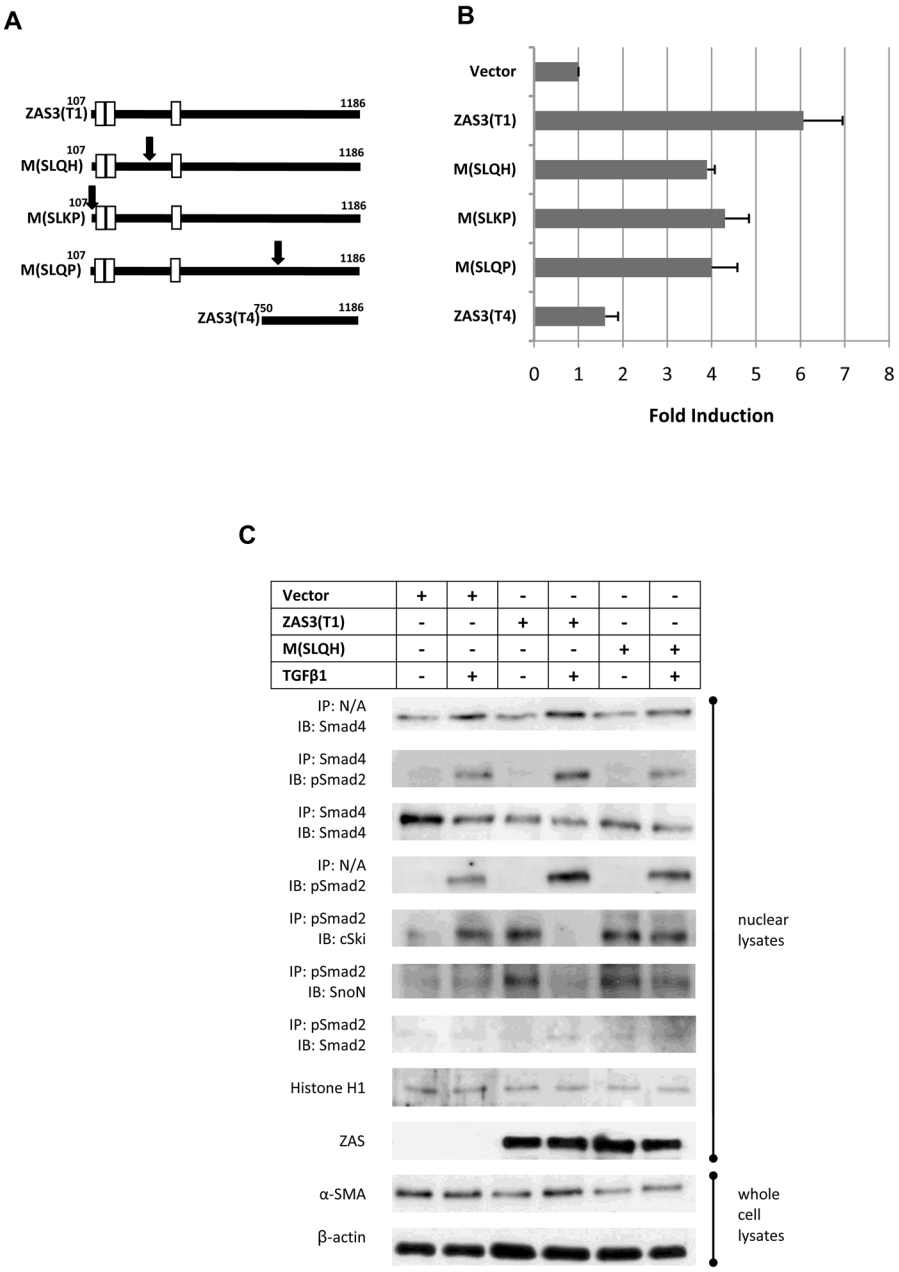

Fig. 4. Site directed mutations attenuate ZAS3 mediated signal accentuation, interactions with the Smad proteins, and induction of α-SMA

A, Site-directed mutations were introduced to ZAS3(T1). The residues mutated to alanine are indicated by parenthesis, the locations are marked by arrows. Single- (▯), and double-zinc finger (▯▯) regions are also indicated. B, HEK293 cells were co-transfected with mutated constructs (0.5 μg/well) and 4×SBE-luc reporter (0.3 μg/well). Medium was replaced 24 h post-transfection with DMEM (10% FBS) and cells were stimulated with TGFβ1 (2 ng/mL) for 24 h prior to measurement of luciferase activity. C, Physical interactions between Smad proteins and Ski/SnoN in nuclear extracts from mutant ZAS3 transfected (0.5 μg/well), TGFβ1 (2 ng/mL) stimulated (1 h), HEK293 cells. For α-SMA induction, cells were transfected with indicated ZAS3 construct, treated with TGFβ1 (2 ng/mL) for 24 h prior to assessing levels in whole cell lysates by Western blotting; β-actin levels were used for an input control.

Luciferase values are means from at least three independent experiments ± S.D., co-IP results are representative of multiple independent experiments.

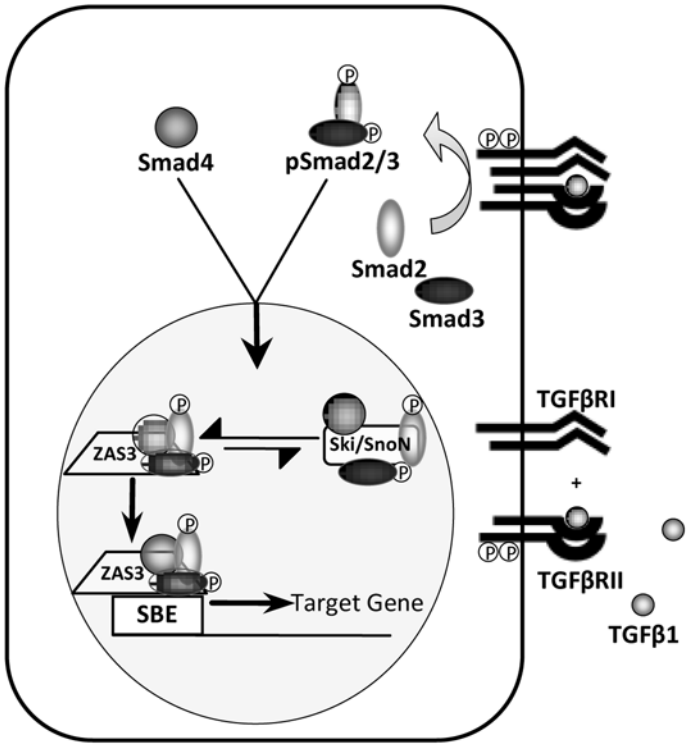

Fig. 5. Proposed mechanism of ZAS3 mediated TGFβ1 signal accentuation

In our model, ZAS3 competes with Ski/SnoN binding to the Smad complex. Binding of the Smad complex to ZAS3 enables association with the SBE and transcription of target genes. Association between the Smad complex and Ski/SnoN results in complex disruption and inhibition of transcription. ZAS3 transfection shifts the equilibrium in favor of ZAS3-Smad complex association, this results in TGFβ signal accentuation.