TMEPAI, a transmembrane TGF-β-inducible protein, sequesters Smad proteins from active participation in TGF-β signaling

Yukihide Watanabe[1], Susumu Itoh[1,4], Toshiyasu Goto[2], Eriko Ohnishi[2], Masako Inamitsu[1], Fumiko Itoh[1], Kiyotoshi Satoh[2], Eliza Wiercinska[3], Weiwen Yang[1], Liang Shi[1], Aya Tanaka[1], Naoko Nakano[1], A Mieke Mommaas[3], Hiroshi Shibuya[2], Peter ten Dijke[3] and Mitsuyasu Kato[1]

[1]Department of Experimental Pathology, Graduate School of Comprehensive Human Sciences, University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan, [2]Department of Molecular Cell Biology, Medical Research Institute, Tokyo Medical and Dental University, 1-5-45, Yushima, Bunkyo-ku, Tokyo 113-8510, Japan, [3]Department of Molecular Cell Biology and Centre for Biomedical Genetics, Leiden University Medical Center, Postzone S-1-P, Postbus 9600, 2300 RC, Leiden, The Netherlands

Running title: Negative regulation of TGF-β/Smad signaling

[4]Corresponding author:
Department of Experimental Pathology,
Graduate School of Comprehensive Human Sciences,
University of Tsukuba, 1-1-1 Tennodai, Tsukuba, Ibaraki 305-8575, Japan
Tel/Fax: +81-29-853-3944; Email: sitoh@md.tsukuba.ac.jp

Summary

Transforming growth factor-β (TGF-β) is a multifunctional cytokine of key importance for controlling embryogenesis and tissue homeostasis. How TGF-β signals are attenuated and terminated is not well understood. Here, we show that TMEPAI, a direct target gene of TGF-β signaling, antagonizes TGF-β signaling by interfering with TGF-β type I receptor (TβRI)-induced R-Smad phosphorylation. TMEPAI can directly interact with R-Smads via a Smad interaction motif. TMEPAI competes with Smad anchor for receptor activation for R-Smad binding, thereby sequestering R-Smads from TβRI kinase activation. In mammalian cells, ectopic expression of TMEPAI inhibited TGF-β-dependent regulation of plasminogen activator inhibitor-1, JunB, cyclin-dependent kinase inhibitors and c-myc expression, whereas specific knockdown of TMEPAI expression prolonged duration of TGF-β-induced Smad2 and Smad3 phosphorylation and concomitantly potentiated cellular responsiveness to TGF-β. Consistently, TMEPAI inhibits activin-mediated mesoderm formation in *Xenopus* embryos. Therefore, TMEPAI participates in a negative feedback loop to control the duration and intensity of TGF-β/Smad signaling.

Introduction

Transforming growth factor-β (TGF-β) is a pivotal cytokine that regulates the growth and differentiation of many different cell types. The TGF-β family signals via specific serine/threonine kinase receptors and intracellular signal transducing molecules, termed Smads (Massagué et al. 2005). TGF-β signaling is initiated by ligand binding to TGF-β type II receptor (TβRII), which induces the formation of heteromeric complexes between specific TGF-β type I receptor (TβRI) and TβRII serine/threonine kinases. TβRI [also termed activin receptor-like kinase (ALK)5] is phosphorylated and activated by TβRII. Active TβRI catalyzes the C-terminal serine phosphorylation of receptor-regulated (R-)Smads. Among R-Smads, Smad2 and Smad3 act downstream of TGF-β, activin and nodal type I receptors, whereas Smad1, Smad5 and Smad8 are phosphorylated by bone morphogenetic protein (BMP) type I receptors. After phosphorylation, R-Smads can form a ternary complex with a common-partner Smad4 and then translocate to the nucleus, where they regulate the transcription of target genes (Shi et al. 2003).

TGF-β family members, which include TGF-βs, activins and BMPs, play key roles in embryogenesis and maintenance of tissue homeostasis during adult life. Misregulation of their signaling has been implicated in various diseases including cancer, fibrosis and vascular disorders (Massagué et al. 2000). Because they operate as morphogens, inducing distinct cell fates at different ligand concentrations, duration and intensity are critical determinants in specifying biological responses of TGF-β family members. Each step of the TGF-β signal transduction pathway appears to be subject to both positive and negative regulation. For example, Smad anchor for receptor activation (SARA) has been shown to recruit non-activated Smads to the activated TGF-β receptor complex (Tsukazaki et al.

1998). Among the negative regulators of TGF-β signaling, I-Smads (*ie*, Smad6 and Smad7), Smad ubiquitination regulatory factors (Smurfs), TG-interacting factor (TGIF) and Ski-related novel protein N (SnoN) are direct target genes for TGF-β signaling and contribute to negative feedback loops (Massagué et al. 2005; Itoh and ten Dijke 2007).

Transmembrane prostate androgen-induced RNA (TMEPAI), alternatively termed PMEPA1, STAG1, ERG1.2 or N4wbp4, has been reported to be induced by testosterone or its derivatives and to be implicated in tumorigenesis (Xu et al. 2000 and 2003; Giannini et al. 2003). The transcript of TMEPAI was recently shown to be induced by TGF-β (Brunschwig et al. 2003; Itoh et al. 2003; Levy and Hill 2005). TMEPAI is a type Ib transmembrane protein containing two PY motifs that can interact with HECT-type E3 ubiquitin ligases. TMEPAI has been reported to be involved in p53-mediated apoptosis (Anazawa et al. 2004) and cell growth inhibition (Xu et al. 2003). However, the mechanisms of its action and physiological function are not fully understood. Here, we show that TMEPAI has an essential function in negative regulation of TGF-β signaling in mammalian cells as well as in *Xenopus* embryos.

Results

***TMEPAI* is a direct target gene of the TGF-β signal.** *TMEPAI* expression is potently induced by TGF-β in many different cell lines including AML, HaCaT NIH3T3 and NMuMG cells (Supplementary Fig. 1a, and data not shown). To explore if TMEPAI is a direct target gene of TGF-β signaling, the cells treated with cycloheximide (CHX) 1 h prior to TGF-β stimulation were stimulated with TGF-β for 2 h. The expression of TMEPAI mRNA by TGF-β was elevated in the presence of CHX, indicating that *de novo* protein

synthesis is not required for this response (Supplementary Fig. 1b). Thus, TMEPAI is an immediately early response gene for TGF-β signaling. Consistent with induction of TMEPAI mRNA by TGF-β, the expression of TMEPAI protein after TGF-β stimulation was increased with a delayed peak of its protein expression compared to that of its mRNA expression (Supplementary Fig. 1c). Challenging C2C12 cells with BMP did not induce the expression of *TMEPAI* (Supplementary Fig. 1d), unlike I-Smads whose transcripts are known to be elevated by multiple TGF-β family members (Nakao et al. 1997; Afrakhte et al. 1998).

TMEPAI perturbs TGF-β signaling. To investigate if an immediately early response gene TMEPAI provides feedback signal for TGF-β, we tested the effect of TMEPAI on the Smad-driven transcriptional $(SBE)_4$-luc reporter (Jonk et al. 1998). TMEPAI dose-dependently inhibited TGF-β-induced activation of the $(SBE)_4$-luc reporter, but did not affect BMP-induced $(SBE)_4$-luc activity (Fig. 1a). Since TMEPAI is a type Ib transmembrane protein that might be localized to the plasma membrane, TMEPAI might exert its negative role on TGF-β/Smad signaling by interfering with R-Smad phosphorylation. To examine this possibility, we transfected Smad2 or Smad3 together with constitutively active TβRI, alternatively termed constitutively active ALK5 (ALK5ca), into COS7 cells. Then, immunoprecipitates with anti-Flag antibody were blotted using anti-phospho-Smad antibodies (Persson et al. 1998). TMEPAI markedly reduced phosphorylation of both Smad2 and Smad3 upon ALK5 activation (Fig. 1b and c). Similarly, TMEPAI also blocked activin type I receptor (ALK4)-induced Smad2 phosphorylation (Fig. 1d). On the other hand, BMP type I receptor (ALK6)-induced

Smad1 phosphorylation was not influenced by TMEPAI (Supplementary Fig. 1e). To further confirm that TMEPAI influenced C-terminal Smad2 phosphorylation upon ALK5 activation, Smad2 or Smad2(2SA), whose C-terminal serines targeted by ALK5 kinase are substituted into alanines (Souchelnytskyi et al. 1997), were transfected into COS7 cells in the absence or presence of ALK5ca, metabolically labeled with [^{32}P]orthophosphate and immunoprecipitated. C-terminal Smad2 phosphorylation upon ALK5 activation was efficiently blocked by TMEPAI. As reported (Mori et al. 2004; Sapkota et al. 2006), Smad2(2SA) was weakly phosphorylated upon ALK5 activation, whereas TMEPAI marginally inhibited phosphorylation of Smad2(2SA) (Supplementary Fig. 2). Thus, TMEPAI can efficiently counteract Smad2 phosphorylation catalyzed directly by the ALK5 kinase.

Three variants of the human TMEPAI counterpart have been registered in gene bank (Supplementary Fig. 3a). Two possess the transmembrane domain (TM), whereas the third is deficient in TM domain. Like mouse TMEPAI, ectopic expression of the three human variants blocked TGF-β-induced reporter activity and Smad2 phosphorylation (Supplementary Fig. 3b and c). Thus, all of three human TMEPAI isoforms can inhibit, albeit to different extent, TGF-β signaling. In addition, we observed no functional differences between mouse and human TMEPAI.

To further confirm the significance of TMEPAI in TGF-β signaling, gain-of function and loss-of function studies were performed. Overexpression of TMEPAI in NMuMG cells using an adenoviral expression system prevented the cells from producing plasminogen activator inhibitor (PAI-1), a direct TGF-β target gene, whereas TMEPAI(4A), a non-functional TMEPAI mutant (see Fig. 3e-h), did not affect production

of PAI-1 by TGF-β (Fig. 1e). Cyclin-dependent kinase (cdk) inhibitors (*ex. p15*, *p16* and *p21*) and *JunB* are also known direct target genes for TGF-β signaling (Itoh et al. 1998; Jonk et al. 1998; Pardali and Moustakas 2007). When TMEPAI was expressed in HaCaT cells, the expressions of *JunB* and *cdk* inhibitors upon TGF-β stimulation were decreased (Fig. 1f and Suppl. Fig. 4). In addition, overexpression of TMEPAI in HaCaT cells rescued TGF-β-mediated repression of *c-myc* transcript (Suppl. Fig, 4). Converse to TMEPAI-induced blockage in Smad2 phosphorylation upon ALK5 activation (Fig. 1b and f), the specific knockdown of TMEPAI extended the duration of Smad2 and Smad3 phosphorylation (Fig. 1g). Similarly, ALK5 kinase-mediated Smad2 phosphorylation was augmented when mouse TMEPAI-specific knockdown was performed in NIH3T3 cells (Supplementary Fig. 5a and b). Consistent with these findings, the independent introduction of two distinct shRNAs for human TMEPAI to HaCaT cells enhanced the expression of JunB when cells were stimulated with TGF-β for 10 h (Fig. 1h). To further consolidate these results, siRNAs corresponding to mouse TMEPAI were introduced into NMuMG cells, and ALK5ca-induced PAI-1 expression was measured. PAI-1 produced by NMuMG cells upon ALK5 activation was further enhanced after treatment of cells with TMEPAI-specific siRNA (Supplementary Fig. 5c). These results indicate that TMEPAI is a critically physiological determinant in the attenuation of TGF-β signaling.

TMEPAI interacts with Smad2 and Smad3. I-Smads are known to prevent R-Smads from being phosphorylated by active ALK5 kinase due to its competition with R-Smads for binding to active ALK5 (Nakao et al. 1997). Compared with Smad7, TMEPAI only marginally interacts with the TGF-β receptor complex (Supplementary Fig. 6a), suggesting

that TMEPAI perturbs TGF-β signaling by a mechanism that is distinct from the antagonistic action of Smad7.

Next, we examined the possibility that TMEPAI can directly interact with Smad proteins. Results shown in Fig. 2a revealed that TMEPAI binds to R-Smads (Smad2 and Smad3), but not to Smad4 and Smad7. In addition, we found that R-Smads do not need to be activated for TMEPAI interaction. To be further convinced that TMEPAI can bind to non-phosphorylated Smad2, we examined if GST-Smad2 generated in bacteria can trap *in vitro*-translated TMEPAIΔTM, which has the inhibitory activity of TGF-β signaling (Suppl. Fig. 7a and b, data not shown). Fig. 2b showed that non-phosphorylated Smad2 bound to TMEPAI. However, in these experiments we were unable to confirm that phosphorylated R-Smads can bind to TMEPAI because TMEPAI blocks R-Smad phosphorylation (Fig. 1b and c). To address this issue further, we prepared lysates from cells transfected with either Smad2 alone or with Smad2 and ALK5ca. Subsequently, each lysate was mixed with lysate prepared from cells transfected with TMEPAI alone, immunoprecipitated with anti-Flag antibody, and then analyzed by western blotting with anti-V5 antibody. As seen in Fig. 2c, TMEPAI bound equally to both non-phosphorylated and phosphorylated Smad2. We also explored if TMEPAI interacts with SARA. However, although association between SARA and Smad2 could be seen, no obvious interaction between SARA and TMEPAI was detected (Supplementary Fig. 6b).

After TGF-β stimulation, phosphorylated Smad2 can form a heteromeric complex with Smad4 and then enter the nucleus to regulate target gene expression (Massagué et al. 2005). Consistent with this notion, ectopic TMEPAI expression was found to inhibit complex formation between Smad2 and Smad4 upon ALK5 activation (Fig. 2d). To show

the physiological significance of the association between TMEPAI and Smad2, we investigated the endogenous interaction of TMEPAI with Smad2 in HaCaT cells. Since TMEPAI expression in the absence of TGF-β is low, the cells were treated with TGF-β for 8 h (Suppl. Fig. 1c). As expected, TMEPAI interacted with endogenous Smad2 and phosphorylated Smad2 in HaCaT cells using anti-Smad2 and anti-phosphorylated Smad2 antibodies in the western blot analysis, respectively (Fig. 2e). The interaction between Smad2 and TMEPAI is specific as TMEPAI could not interact with the closely related Smad1 (Supplementary Fig. 1f). Taken together, we concluded that TMEPAI induced by TGF-β preferentially associates with TGF-β/activin R-Smads (*i.e.*, Smad2 and Smad3) to inhibit their signaling responses.

A SIM domain is essential for TMEPAI to block the TGF-β signal. We next explored which domain(s) of TMEPAI contributes to its inhibitory effect on TGF-β signaling. TMEPAI possesses two PY motifs that interact with E3 ubiquitin ligases containing WW domains (Izzi and Attisano, 2004). However, all of the TMEPAI mutants lacking the PY motifs (TMEPAIΔPY1, TMEPAIΔPY2 and TMEPAIΔPY) retained their inhibitory ability of TGF-β signaling (Supplementary Fig. 7a-c). Like human TMEPAI isoform c (Supplementary Fig. 3a-c), mouse TMEPAI lacking its transmembrane domain (TMEPAIΔTM) (Supplementary Fig. 7a), which is mislocalized in cytoplasm (data not shown), could also inhibit, albeit more weakly, the TGF-β-induced reporter activity (Supplementary Fig. 7b). Thus, the integration of TMEPAI in the membrane is not required for its inhibitory effect on TGF-β signaling. To further analyze the domain that is involved in blocking TGF-β signaling, three deletion mutants of TMEPAI from its C-terminus were

generated (Fig. 3a) and tested for their ability to interact with Smad2. The domain from Asn^{171} to Ser^{204} was necessary for its interaction with Smad2 (Fig. 3b). Consistent with the interaction of TMEPAI mutants with Smad2, TMEPAI(1-171) lacking the Smad2-binding domain is unable to block TGF-β receptor-induced responses (Fig. 3c and d).

When we carefully checked the region from Asn^{171} to Ser^{204} in TMEPAI, we identified a peptide sequence (Pro-Pro-Asn-Arg; PPNR) in TMEPAI similar to the Smad interaction motif (SIM) that was originally discovered in the transcriptional factors Milk and Mixer (Randall et al. 2002). To examine the significance of the PPNR sequence in TMEPAI for its inhibitory action of TGF-β signaling, we replaced $P^{178}PNR^{181}$ in TMEPAI with AAAA and termed it TMEPAI(4A) mutant (Fig. 3e). When TMEPAI(4A) was overexpressed in COS7 cells together with Smad2, TMEPAI(4A) no longer interacted with Smad2 (Fig. 3f). Consistent with the results above, TMEPAI(4A) could not block TGF-β-induced Smad2 phosphorylation (Fig. 3g) and reporter activity (Fig. 3h). These results indicate that the SIM domain in TMEPAI is required for its antagonistic effect on TGF-β signaling. To confirm that the SIM domain including adjacent sequences in TMEPAI is sufficient to bind to Smad2, a TMEPAI-derived peptide including SIM domain, which is composed of 50 amino acid residues, was fused to GFP. GFP-SIM was found to interact with Smad2, but not GFP-SIM(4A) (Supplementary Fig. 7d). Thus, this SIM-containing domain alone is sufficient for TMEPAI to bind to Smad2.

If TMEPAI tightly interacts with Smad2, expression of TMEPAI in cells should antagonize Smad2 nuclear translocation after TGF-β treatment. To confirm this possibility, NMuMG cells transiently expressing TMEPAI were stimulated with TGF-β for 1 h. As shown in Fig. 3i, TGF-β-induced Smad2 nuclear accumulation was blocked upon ectopic

TMEPAI expression (white arrows). On the other hand, TMEPAI(4A), defective in Smad2 binding, did not affect this TGF-β-induced response (Fig. 3j). Moreover, we found that the TMEPAI is mainly localized in Golgi apparatus as well as in endosomes where SARA can be present (see below) (Supplementary Fig. 8a-c), whereas only few particles corresponding to the TMEPAI molecule were detected on the plasma membrane (data not shown). Using immunofluorescence, we observed a co-localization of TMEPAI with Smad2 upon TGF-β (Supplementary Fig. 8d). Furthermore, electron microscopy revealed that Smad2 can be located in endosomes with TMEPAI (Supplementary Fig. 8e, f).

TMEPAI competes with SARA for Smad2 binding. We used several Smad2 mutants to examine which domain(s) of Smad2 is required for interaction with TMEPAI. The MH2 domain in Smad2 was found to be sufficient and required for association with TMEPAI (Supplementary Fig. 9). Previously it was reported that Trp^{368} in the MH2 domain of Smad2 is critical for Smad2 to bind to the SIM domain of Milk and Mixer (Randall et al. 2002). Therefore, we explored whether Trp^{368} in Smad2 is necessary for the interaction with TMEPAI. As seen in Fig. 4a, Smad2(W368A) mutant had no ability to interact with TMEPAI. Consistently, the GST-pulldown experiment showed that *in vitro* [^{35}S]-labeled TMEPAIΔTM specifically associated with GST-Smad2, but not with GST-Smad2(W368A) (Fig. 2b). The tryptophan residue critical for TMEPAI interaction is conserved in Smad2 and Smad3, but not in other Smads. This provides an explanation for the reason why TMEPAI does not influence BMP signaling.

The Smad binding domain (SBD) in SARA is necessary for SARA's function in recruiting Smad2 to ALK5 (Tsukazaki et al. 1998). The amino acid sequence of the rigid

coil in SARA SBD is similar to that of the SIMs in Milk and Mixer (Randall et al. 2002), but the flanking amino acid sequence other than PPNR in TMEPAI has no similarity to SARA SBD. Ectopic expression of SARA(SBD) (Zhao and Hoffmann 2006), however, abrogated the interaction between TMEPAI and Smad2 (Fig. 4b). In addition, TMEPAI could disrupt the interaction between Smad2 and SARA(SBD) (Fig. 4c). Thus, the SIM domain is required for TMEPAI to block interaction between Smad2 and SARA.

The electron microscopy showed that TMEPAI is localized in endosomes (Supplementary Fig. 8b, c, e, f), which prompted us to investigate if TMEPAI co-localizes with SARA in cells. We have already reported that the FYVE domain of SARA, termed SARA(FYVE), co-localizes with SARA in early endosomes (Itoh et al. 2002). Therefore, TMEPAI/V5 was transfected in 911 cells with GFP-SARA(FYVE) instead of SARA because of low expression of SARA in cells. Both TMEPAI and GFP-SARA(FYVE) showed co-localization with punctate staining (Fig. 4d). Consistent with our immunofluorescence microscopy analysis, TMEPAI was localized on the endosome-like structure where GFP-FYVE was also positive using the electron microscopy (Fig. 4e). Although we expected that most of TMEPAI neighbor to GFP-SARA(FYVE) on the endosome-like structure, only a part of small dots corresponding to TMEPAI (arrows) were closed to the eodosome membrane where the big dots corresponding to GFP-SARA(FYVE) are located. There is a possibility that the localization of SARA was not complete identical to that of GFP-SARA(FYVE) in endosomes. Thereby, we might not be able to observe enough colocalization between TMEPAI and GFP-SARA(FYVE) with the electron microscopy. We further examined if SARA could rescue TMEPAI-mediated repression of the reporter activity induced by TGF-β. As expected, TGF-β-induced

reporter activity inhibited by TMEPAI could be relived by SARA dose-dependently (Fig. 4f). Consistent with the recovery of the reporter activity, SARA restored TMEPAI-mediated repression of Smad2 phosphorylation upon ALK5 activation (Fig. 4g). SARA has been reported to require Trp^{368} and Asn^{381} residues in Smad2 for its efficient binding to Smad2 (Wu et al. 2000). Indeed, Smad2(W368A) still has the ability to interact with SARA (data not shown). Occupation of Trp^{368} in Smad2 by TMEPAI possibly affects the complex formation between SARA and Smad2, and therefore SARA SBD might not reach Asn^{381} in Smad2. These results indicate that TMEPAI interferes with the SARA-Smad2 complex formation. Thus, in the presence of TMEPAI, SARA cannot efficiently recruit Smad2 to TβRI upon TGF-β stimulation.

TMEPAI inhibits activin-induced mesoderm formation in *Xenopus* embryos. On the basis of the studies in cultured mammalian cells described above, we predicted that TMEPAI may inhibit activin signaling during *Xenopus* embryogenesis. For that purpose, we isolated a *Xenopus* counterpart for mammalian TMEPAI. *Xenopus* TMEPAI (xTMEPAI) shares 77% and 70% amino acid sequence identity with human and mouse TMEPAI, respectively (Supplementary Fig. 10a). However, the TM, SIM and PY motifs in TMEPAI of the 3 species are almost identical. When the expression pattern of TMEPAI during embryonic development in *Xenopus* was investigated by the methods of RT-PCR and *in situ* hybridizaiton, TMEPAI was ubiquitously expressed at all stages that we examined (Supplementary Fig. 10b and c). We analyzed patterning phenotypes caused by overexpression of xTMEPAI in *Xenopus* embryos. When the endogenous activin signaling pathway is inactivated in early *Xenopus* embryos, mesoderm fails to form

(Hemmati-Brivanlou and Melton 1992; Chang et al. 1997). Similarly, microinjection of mRNA encoding xTMEPAI into the dorsal marginal zone (DMZ) of 4-cell embryos inhibited mesoderm formation. Specifically, head and tail structures were absent or severely deficient in 96% of the injected embryos (n=25) (Fig. 5a). On the other hand, the injected embryos showed hardly any deficiencies when the same amount of xTMEPAI mRNA was injected into the animal pole or the ventral marginal zone (VMZ) (data not shown). Activin is one of the endogenous mesoderm-inducing molecules (Kessler and Melton, 1994). To examine whether xTMEPAI blocks activin-induced mesoderm formation *in vivo*, we performed the *Xenopus* animal cap assay. Activin induced expression of Xbra, a pan-mesodermal marker, and gsc, a dorsal mesodermal marker. When xTMEPAI mRNA was injected into the animal poles of 2-cell embryos, xTMEPAI dose-dependently blocked the expression of Xbra and gsc markers (Fig. 5b). Moreover, the injection of xTMEPAI mRNA into the DMZ of 4-cell embryos also prevented the endogenous expression of both Xbra and gsc markers in a dose-dependent manner (Fig. 5c). These results demonstrate that xTMEPAI can perturb activin-mediated mesoderm induction.

To further consolidate the inhibitory effect of xTMEPAI in *Xenopus* embryos, we examined the molecular effects of xTMEPAI knockdown by monitoring expression of activin-induced marker genes in animal cap cells. To prove the specificity of xTMEPAI morpholino oligonucleotide (MO), xTMEPAI mRNA was injected into the animal blastomeres in *Xenopus* embryos. As expected, TMEPAI MO specifically reduced the expression of ectopic xTMEPAI (Supplementary Fig. 10d). The injection of TMEPAI MO augmented expression of Xbra and gsc in comparison with that of the control MO

(scramble MO) or the mock-injected (Fig. 5d). On the other hand, FGF-induced expression of Xbra in the animal caps was not influenced by the injection of xTMEPAI MO (Supplementary Fig. 10e). Above evidence supports the view that TMEPAI blocks the activin signaling pathway in *Xenopus* embryo.

Discussion

Positive and negative signals are equally critical for regulation of the TGF-β/Smad signaling pathway (Massagué et al. 2005). Disruption of the balance between positive and negative regulation in TGF-β signaling can lead to various diseases (Massagué et al. 2000). We have herein presented evidences demonstrating that TMEPAI, a transmembrane protein, plays a role in termination of TGF-β signaling. Since TMEPAI is a direct early target gene for TGF-β signaling, we conceived the possibility that TMEPAI acts like I-Smads in a negative feedback loop. Indeed, Smad2 phosphorylation as well as Smad2 nuclear accumulation reached the highest peak 1-2 h after TGF-β stimulation, and then both levels gradually fell down as TMEPAI and Smad7 proteins went up (Suppl. Fig. 11a-c). The inhibitory abilities which TMEPAI possesses were specific for TGF-β and activin pathways, but not for the closely related BMP pathway. Thus, an interesting question arose how TMEPAI achieves such a high specificity. Although we initially thought that the PY motifs in TMEPAI were involved in its inhibitory effects on TGF-β signaling, our mutants lacking the PY motifs displayed inhibitory actions comparable with those of wild-type TMEPAI (Supplementary Fig. 7a-c). Hence, we looked for other motif(s) in TMEPAI, which was involved in its inhibitory action. We found that the SIM domain in TMEPAI (PPNR) was critical for sequestering Smad2 and Smad3 from active

participation in TGF-β signaling. In our loss-of-function study, Smad2 phosphorylation by TGF-β extended longer in HaCaT cells expressing TMEPAI shRNA than Smad3 phosphorylation by TGF-β (Fig. 1g). Although the affinity between ectopic Smad2 and ectopic TMEPAI was almost equal to that between ectopic Smad3 and ectopic TMEPAI in COS7 cells (Fig. 2a), the affinity between Smad2 and TMEPAI might be higher in the physiological condition than that between Smad3 and TMEPAI. We will further investigate this point of view in future. As previously shown for the SIM domain in Mixer (Randall et al. 2004), the SIM domain in TMEPAI also showed comparable affinity to both non-phosphorylated and phosphorylated Smad2. Thus, TMEPAI can trap not only non-phosphorylated Smad2 and Smad3 but also TβRI kinase-activated Smad2 and Smad3 after ligand stimulation in order to efficiently terminate TGF-β signaling. Binding of Smad2 and Smad3 to TMEPAI is mutually exclusive with binding to SARA. TMEPAI sequesters Smad2 and Smad3 from SARA so that SARA cannot recruit Smad2 and Smad3 to the activated TGF-β receptor complex. Besides, we might also consider the possibility that TMEPAI captures phosphorylated Smad2 and Smad3 to prevent their nuclear translocation (Fig. 6).

Reduced levels of endogenous SARA in mouse embryonic fibroblasts (MEFs) from Hgs-knockout mice (Kobayashi et al. 2005) or in *Xenopus* embryos did not alter TGF-β or activin signal (data not shown). It has been reported that like SARA, Hgs which possesses a FYVE domain also has the attribute of acting as a scaffold protein for TGF-β signaling (Miura et al. 2000). Furthermore, ELF (Tang et al. 2003b) and Filamin (Sasaki et al. 2001) are also known as scaffold proteins in TGF-β signaling. However, Hgs, ELF and Filamin do not have a SIM domain in their structures, suggesting that TGF-β signaling

involving these molecules might be insensitive to TMEPAI. As another possibility, SARA-like scaffold protein(s) which also possesses FYVE and SBD in its structure might compensate for lack of SARA. Importantly, over-expression of SARA restored suppression of TGF-β signal by TMEPAI (Fig. 4f and g). Up to now, there have been no clear evidences that SARA positively regulates TGF-β signal although SARA mutants lacking functional domains (*ex.* FYVE or SBD domain) could block TGF-β signal (Tsukazaki et al. 1998). Our current results obviously demonstrate that SARA is involved in TGF-β signaling through competition with TMEPAI. It might be possible that the fine balance between SARA and TMEPAI hampers aberrant TGF-β signaling in cells. SIM domains in the transcription factors Milk and Mixer are known to compete with the SBD in SARA for binding to Smad2, but the physiological significance of the competition between Milk or Mixer and SARA for R-Smad binding remains veiled because Milk and Mixer are nuclear proteins (Randall, 2002). SARA and TMEPAI compete for R-Smad binding as they can be localized in the same organelle in cells. Our present results elucidated a physiological significance of the SIM domain to compete with SARA for R-Smad binding in the TGF-β pathway.

In addition, we found no evidence that TMEPAI affects BMP signaling, and thus TMEPAI seems to be a specific negative regulator of TGF-β signaling. Recently, endofin was found to be a Smad anchor for receptor activation in BMP signaling (Shi et al. 2007). It is possible that there is a TMEPAI-like molecule(s) that competes with endofin for Smad1 binding.

In *Xenopus* embryos, injection of TMEPAI mRNA into the VMZ, in which BMP signaling is preferentially transduced, did not alter the tadpole's phenotype (data not

shown) although ventralization with no eyes and no tail was promoted by injection of TMEPAI into the DMZ, where the activin signal is predominantly active. Our observation from *Xenopus* model confirmed the results obtained in mammalian cultured cells, that TMEPAI displays specific inhibitory actions on the TGF-β/activin pathway through sequestering Smad2 and Smad3 from TGF-β receptor activation.

We also observed high expression of TMEPAI in tumor tissues from patients with breast (Suppl. Fig. 12a), lung and prostate cancers (data not shown) as reported (Giannini et al. 2003). Since tumor cells exploit TGF-β signaling to maintain motility or malignant behavior, genetically inactive mutations in TGF-β receptors or Smads possibly correlate not only with loss of the TGF-β-mediated cytostatic response but also with loss of the malignant phenotype (Derynck et al. 2001). Thus, temporal interruption of TGF-β signaling by overexpression of TMEPAI might give an advantage to tumor cells. However, we could not conclude whether or not increase of TMEPAI protein is correlated with active TGF-β signaling (data not shown). Since the expression of TMEPAI has been known to be induced by serum, EGF, active Wnt signaling as well as overexpression of mutant p53 (Xu et al. 2000; Giannini et al. 2003; Anazawa et al. 2004; Muñoz et al. 2006), deregulated signal pathway(s), in stead of TGF-β signal, in certain tumors might raise the expression of TMEPAI. In $Apc^{Min/+}$ mice developing multiple intestinal adenoma with biallelic inactivating mutation in the *Apc* gene (Moser et al. 1990; Su et al. 1992), our immunohistochemical results clearly demonstrated that TMEPAI is specifically expressed in highly proliferative (or Ki-67-positive) adenoma with activated Wnt signaling (or nuclear accumulation of β-catenin) (Suppl Fig. 12b). In the genetically modified mouse model, the inactivation of TGF-β receptor together with activation of the Wnt/β-catenin

signaling pathway accelerates malignant progression of intestinal neoplasms (Muñoz et al. 2006). It would be interesting to examine if TMEPAI is implicated in malignancy of colorectal tumors carrying a mutation(s) in the *Apc* or *β-catenin* gene. Furthermore, a question arises if transcriptional cooperation between TGF-β and Wnt signalings affects TMEPAI gene regulation because high expression of TMEPAI in intestinal adenoma is suspected to develop tumorigenicity.

In conclusion, TMEPAI is a TGF-β/activin-specific inhibitor which preferentially perturbs recruitment of Smad2 or Smad3 to TβRI by SARA. Thus, TMEPAI can limit the duration of Smad phosphorylation in a negative feedback mechanism. Loss of TGF-β responsiveness via genetic hereditary mutation, somatic mutation, or aberrant expression of components for TGF-β signaling is linked to tumorigenicity or inheritable disorders. From this point of view, TMEPAI might become a potential target for therapeutic intervention.

Experimental Procedures

Expression Plasmids –Constructs used in this study were described in Supplemental data.

Cell Culture – NMuMG, NIH3T3, HaCaT, 911 and COS7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal calf serum (FCS, Bio-west) and non-essential amino acids (NEAA, Invitrogen). HepG2 cells were maintained in minimum essential medium (Sigma) containing 10%FCS, NEAA and sodium pyruvate. MCF10A1 cells were grown according to Tang et al. (2003a).

Electron microscopy - MCF10A1 cells were transfected with TMEPAI/V5 and GFP-SARA(FYVE) (Itoh et al. 2002) and cultured for 40 h. The detail procedures were described in Supplemental data.

RT-PCR, RNA preparation, transfection, reporter assay, immunoprecipitation, western blotting,PAI-1 production, immunofluorescence and GST pulldown– RT-PCR, RNA preparation, transient transfection, reporter assay, immunoprecipitation, western blotting, immunofluorescence and GST pulldown were performed as previously described (Itoh et al. 2000, 2003 and 2004; Noda et al. 2006). In all reporter assays, β-galactosidase expression vector pCH110 (GE Healthcare) was used as an internal control. The experiments were carried out in triplicate at least twice. All values represent the mean ± SD (n=3). To generate antibodies against TMEPAI, rabbits were immunized with the synthetic peptide (LSDGEEPPPYQGPC). In the GST pull-down assay, ^{35}S-labeled TMEPAIΔTM was generated by mixture of TNT T7 coupled reticulocyte lysate system (Promega), Tran[^{35}S]-Label (0.37 MBq/ml; MP Biochemicals) and pcDNA3-TMEPAIΔTM as a template.

Lentiviral shRNAs for TMEPAI- Non-targeting shRNA (SHC002), TMEPAI shRNA#2 (TRC332) and #5(TRC335) lentiviral vectors (Sigma) were used for knockdown of TMEPAI.

Microinjection of synthetic mRNA and RT-PCR analysis for Xenopus embryos– Unfertilized eggs were collected and fertilized *in vitro* as previously described (Suzuki et al. 1997). Animal cap assays were carried out as previously described (Yamamoto et al. 2000). PCR primer sets were described in Supplemental data.

Acknowledgements

This research was supported by Grants-in-aid for Scientific Research (17390073, 20012007 and 21590328) (M. K. and S. I.); a grant of the Genome Network Project (M. K.) from the Ministry of Education, Culture, Sports, Science and Technology; Grant-in-Aid for JSPS Fellows from Japan Society for the Promotion of Science (Y. W. and F. I.); a grant of Long-range Research Initiative (LPI) by Japan Chemical Industry Association (JCIA) (M. K.); AstraZeneca Research Grant 2004 (S. I.); Kowa Life Science Foundation (S. I.); Kato Memorial Bioscience Foundation (S. I.); and Dutch Cancer Society EC 6th framework STREP Tumor-Host Genomics and Centre for Biomedical Genetics (P. t-D.). We thank J. Onderwater for technical assistance in electron microscopy experiments, Dr. F. Miyamasu for excellent English proofreading, Dr. K. Miyazono for adenoviral ALK5ca, Dr. K. Iwata for TGF-β3, and Dr. K. Sampath for BMP-6.

References

Afrakhte, M., Morén, A., Jossan, S., Itoh, S., Sampath, K., Westermark, B., Heldin, C.-H., Heldin, N.-E., and ten Dijke, P. (1998) Induction of Smad6 and Smad7 mRNA by TGF-β family members. Biochem. Biophys. Res. Commun. *249*, 505-511.

Anazawa, Y., Arakawa, H., Nakagawa, H., and Nakamura, Y. (2004) Identification of STAG1 as a key mediator of a p53-dependent apoptotic pathway. Oncogene *23*, 7621-7627.

Brunschwig, E.B., Wilson, K., Mack, D., Dawson, D., Lawrence, E., Willson, J.K., Lu, S., Nosrati, A., Rerko, R.M., Swinler, S. et al. (2003) *PMEPA1*, a transforming growth factor-β-induced marker of terminal colonocyte differentiation whose expression is maintained in primary and metastatic colon cancer. Cancer Res. *63*, 1568-1575.

Chang, C., Wilson, P.A., Mathews, L.S., and Hemmati-Brivanlou, A. (1997) A *Xenopus* type I activin receptor mediates mesodermal but not neural specification during embryogenesis. Development *124*, 827-837.

Derynck, R., Akhurst, R.J., and Balmain, A. (2001) TGF-β signaling in tumor suppression and cancer progression. Nat. Genet. *29*, 117-129.

Giannini, G., Ambrosini, M.I., Di Marcotullio, L., Cerignoli, F., Zani, M., MacKay, A.R., Screpanti, I., Frati, L., and Gulino, A. (2003) EGF- and cell cycle-regulated *STAG1/PMEPA1/ERG1.2* belongs to a conserved gene family and is overexpressed and amplified in breast and ovarian cancer. Mol. Carcinog. *38*, 188-200.

Hemmati-Brivanlou, A., and Melton, D.A. (1992) Truncated activin receptor inhibits

mesoderm induction and formation of axial structures in *Xenopus* embryos. Nature *359*, 609-614.

Itoh, F., Divecha, N., Brocks, L., Oomen, L., Janssen, H., Calafat, J., Itoh, S., and ten Dijke, P. (2002) The FYVE domain in Smad anchor for receptor activation (SARA) is sufficient for localization of SARA in early endosomes and regulates TGF-β/Smad signalling. Genes Cells *7*, 321-331.

Itoh, F., Itoh, S., Goumans, M.-J., Valdimarsdottir, G., Iso, T., Dotto, G.P., Hamamori, Y., Kedes, L., Kato, M., and ten Dijke, P. (2004) Synergy and antagonism between Notch and BMP receptor signaling pathways in endothelial cells. EMBO J. *23*, 541-551.

Itoh, S., Ericsson, J., Nishikawa, J., Heldin, C.-H., and ten Dijke, P. 2000. The transcriptional co-activator P/CAF potentiates TGF-β/Smad signaling. Nucleic Acids Res. *28*, 4291-4298.

Itoh, S., Landström, M., Hermansson, A., Itoh, F., Heldin, C.-H., Heldin, N.-E., and ten Dijke, P. 1998. Transforming growth factor β1 induces nuclear export of inhibitory Smad7. J. Biol. Chem. *273*, 29195-29201.

Itoh, S., Thorikay, M., Kowanetz, M., Moustakas, A., Itoh, F., Heldin, C.-H., and ten Dijke, P. 2003. Elucidation of Smad requirement in transforming growth factor-β type I receptor-induced responses. J. Biol. Chem. *278*, 3751-3761.

Itoh, S., and ten Dijke, P. (2007) Negative regulation of TGF-β receptor/Smad signal transduction. Curr. Opin. Cell Biol. *19*, 176-184.

Izzi, J., and Attisano, L. (2004) Regulation of the TGFβ signalling pathway by ubiquitin-mediated degradation. Oncogene *23*, 2071-2078.

Jonk, L.K.C., Itoh, S., Heldin, C.-H., ten Dijke, P., and Kruijer, W. (1998) Identification and functional characterization of a Smad binding element (SBE) in the *Jun B* promoter that acts as a transforming growth factor β, activin and bone morphogenetic protein-inducible enhancer. J. Biol. Chem. *273*, 21145-21152.

Kessler, D.S., and Melton, D.A. (1994) Vertebrate embryonic induction: mesodermal and neural patterning. Science *266*, 596-604.

Kobayashi, H., Tanaka, N., Asao, H., Miura, S., Kyuuma, M., Semura, K., Ishii, N., and Sugamura, K. (2005) Hrs, a mammalian master molecule in vesicular transport and protein sorting, suppresses the degradation of ESCRT proteins signal transducing adaptor molecule 1 and 2. J. Biol. Chem. *280*, 10468-10477.

Levy, L., and Hill, C.S. (2005) Smad4 dependency defines two classes of transforming growth factor β (TGF-β) target genes and distinguishes TGF-β-induced epithelial-mesenchymal transition from its antiproliferative and migratory responses. Mol. Cell. Biol. *25*, 8108-8125.

Massagué, J., Blain, S. W., and Lo, R.S. (2000) TGFβ signaling in growth control, cancer, and heritable disorders. Cell *103*, 295-309.

Massagué, J., Seoane, J., and Wotton, D. (2005) Smad transcription factors. Genes Dev. *19*, 2783-2810.

Miura, S., Takeshita, T., Asao, H., Kimura, Y., Murata, K., Sasaki, Y., Hanai, J.I., Beppu, H., Tsukazaki, T., Wrana, J.L. et al. (2000) Hgs (Hrs), a FYVE domain protein, is involved in Smad signaling through cooperation with SARA. Mol. Cell. Biol. *20*, 9346-9355.

Mori, S., Matsuzaki, K., Yoshida, K., Furukawa, F., Tahashi, Y., Yamagata, H., Sekimoto,

G., Seki, T., Matsui, H., Nishizawa, M. et al. (2004) TGF-β and HGF transmit the signals through JNK-dependent Smad2/3 phosphorylation at the linker regions. Oncogene *23*, 7416-7429.

Moser, A.R., Pitot, H.C., and Dove, W.F. (1990) A dominant mutation that predisposes to multiple intestinal neoplasia in the mouse. Science *247*, 322–324.

Muñoz, N.M., Upton, M., Rojas, A., Washington, M.K., Lin, L., Chytil, A., Sozmen, E.G., Madison, B.B., Pozzi, A., Moon, R.T. et al. (2006) Transforming growth factor β receptor type ii inactivation induces the malignant transformation of intestinal neoplasms initiated by *Apc* mutation. Cancer Res. *66*, 9837-9844.

Nakao, A., Afrakhte, M., Morén, A., Nakayama, T., Christian, J.L., Heuchel, R., Itoh, S., Kawabata, M., Heldin, N.-E., Heldin, C.-H. et al. (1997) Identification of Smad7, a TGF-β-inducible antagonist of TGF-β signaling. Nature *389*, 631-635.

Noda, D., Itoh, S., Watanabe, Y., Inamitsu, M., Dennler, S., Itoh, F., Koike, S., Danielpour, D., ten Dijke, P., and Kato, M. (2006) ELAC2, a putative prostate cancer susceptibility gene product, potentiates TGF-β/Smad-induced growth arrest of prostate cells. Oncogene *25*, 5591-5600.

Pardali, K., and Moustakas, A. (2007) Actions of TGF-β as tumor suppressor and pro-metastatic factor in human cancer. Biochim. Biophys. Acta *1775*, 21-62.

Persson, U., Izumi, H., Souchelnytskyi, S., Itoh, S., Grimsby, S., Engström, U., Heldin, C.-H., Funa, K., and ten Dijke, P. (1998) The L45 loop in type I receptors for TGF-β family members is a critical determinant in specifying Smad isoform activation. FEBS Lett. *434*, 83-87.

Randall, R.A., Germain, S., Inman, G.J., Bates, P.A., and Hill, C.S. (2002) Different

Smad2 partners bind a common hydrophobic pocket in Smad2 via a defined proline-rich motif. EMBO J. *21*, 145-156.

Randall, R.A., Howell, M., Page, C.S., Daly, A., Bates, P.A., and Hill, C.S. (2004) Recognition of phosphorylated-Smad2-containing complexes by a novel Smad interaction motif. Mol. Cell. Biol. *24*, 1106-1121.

Sapkota, G., Knockaert, M., Alarcón, C., Montalvo, E., Brivanlou, A.H., and Massagué, J. (2006) Dephosphorylation of the linker regions of Smad1 and Smad2/3 by small C-terminal domain phosphatases has distinct outcomes for bone morphogenetic protein and transforming growth factor-β pathways. J. Biol. Chem. *281*, 40412-40419.

Sasaki, A., Masuda, Y., Ohta, Y., Ikeda, K., and Watanabe, K. (2001) Filamin associates with Smads and regulates transforming growth factor-β signaling. J. Biol. Chem. *276*, 17871-178717.

Shi, W., Chang, C., Nie, S., Xie, S., Wan, M., and Cao, X. (2007) Endofin acts as a Smad anchor for receptor activation in BMP signaling. J. Cell Sci. *120*, 1216-1224.

Shi, Y., and Massagué, J. 2003. Mechanisms of TGF-β signaling from cell membrane to the nucleus. Cell *113*, 685-700.

Souchelnytskyi, S., Tamaki, K., Engström, U., Wernstedt, C., ten Dijke, P., and Heldin, C.-H. (1997) Phosphorylation of Ser^{465} and Ser^{467} in the C terminus of Smad2 mediates interaction with Smad4 and is required for transforming growth factor-β signaling. J. Biol. Chem. *272*, 28107-28115.

Su, L.K., Kinzler, K.W., Vogelstein, B., Preisinger, A.C., Moser, A.R., Luongo, C., Gould, K.A., and Dove, W.F. (1992) Multiple intestinal neoplasia caused by a mutation in

the murine homolog of the APC gene. Science *256*, 668–670.

Suzuki, A., Kaneko, E. Maeda, J., and Ueno, N. (1997) Mesoderm induction by BMP-4 and -7 heterodimers. Biochem. Biophys. Res. Commun. *232*, 153–156.

Tang, B., Vu, M., Booker, T., Santner, S.J., Miller, F.R., Anver, M.R., and Wakefield, L.M. (2003a) TGF-β switches from tumor suppressor to prometastatic factor in a model of beast cancer progression. J. Clin. Invest. *112*, 1116-1124.

Tang, Y., Katuri, V., Dillner, A., Mishra, B., Deng C.-X., and Mishra, L. (2003b) Disruption of transforming growth factor-β signaling in ELF β-spectrin-deficient mice. Science *299*, 574-577.

Tsukazaki, T., Chiang, T.A., Davison, A.F., Attisano, L., and Wrana, J.L. (1998) SARA, a FYVE domain protein that recruits Smad2 to TGFβ receptor. Cell *95*, 779-791.

Wu, G., Chen, Y.G., Ozdamar, B., Gyuricza, C.A., Chong, P.A., Wrana, J.L., Massagué, J., and Shi, Y. (2000) Structural basis of Smad2 recognition by the Smad anchor for receptor activation. Science *287*, 92-97.

Xu, L.L., Shanmugam, N., Segawa, T., Sesterhenn, I.A., McLeod, D.G., Moul, J.W., and Srivastava, S. (2000) A novel androgen-regulated gene, *PMEPA1*, located on chromosome 20q13 exhibits high level expression in prostate. Genomics *66*, 257-263.

Xu, L.L., Shi, Y., Petrovics, G., Sun, C., Makarem, M., Zhang, W., Sesterhenn, I.A., McLeod, D.G., Sun, L., Moul, J.W., and Srivastava, S. (2003) PMEPA1, an androgen-regulated NEDD4-binding protein, exhibits cell growth inhibitory function and decreased expression during prostate. Cancer Res. 63, 4299-4304.

Yamamoto, T.S., Takagi, C., and Ueno, N. (2000) Requirement of *Xmsx-1* in the

BMP-triggered ventralization of *Xenopus* embryos. Mech. Dev. *91*, 131–141.

Zhao, B.M., and Hoffmann, F.M. (2006) Inhibition of transforming growth factor-β1-induced signaling and epithelial-to-mesenchymal transition by the Smad-binding peptide aptamer. Trx-SARA. Moll. Biol. Cell. *17*, 3819-3831.

Figure legends

Fig. 1. TMEPAI blocks TGF-β signaling. (a) Effect of TMEPAI on TGF-β- or BMP-induced reporter activity. Different doses of TMEPAI were co-transfected with $(SBE)_4$-luc in HepG2 cells with or without 5 ng/ml TGF-β or 25 ng/ml BMP-6 for 18 h. (b, c) Inhibition of ALK5ca-induced (b) Smad2 and (c) Smad3 phosphorylation by TMEPAI. COS7 cells were transfected with indicated plasmids. To show phosphorylation of Smad2 or Smad3 upon ALK5 activation, the cell lysates were immunoprecipitated with anti-Flag M5 antibody and then analyzed by western blotting with anti-phospho-Smad2 (PS2) or anti-phospho-Smad1/3 antibody (PS1/3). (d) TMEPAI perturbs ALK4ca-induced Smad2 phosphorylation. COS7 cells were transfected with indicated plasmids. To show phosphorylation of Smad2 upon ALK4 activation, the cell lysates were immunoprecipitated with anti-Flag M5 antibody and then analyzed by western blotting with PS2. ALK5ca was used as a positive control. (e) TMEPAI blocks TGF-β-induced PAI-1 production. NMuMG cells were infected with GFP-expressing adenovirus (Ad-GFP), TMEPAI- (Ad-GFP-TMEPAI) or TMEPAI(4A)-expressing adenoviruses (Ad-GFP-TMEPAI(4A)). Cells were treated with 5 ng/ml TGF-β for 6 h. Three hours before lysis of cells, Tran[^{35}S]-Label was added to the medium. (f) TGF-β-induced expression of JunB and cdk inhibitor p21 is inhibited by TMEPAI. HaCaT cells were infected with Ad-GFP or Ad-GFP-TMEPAI. Cells were stimulated with 5 ng/ml TGF-β for 4 h. Subsequently, total cell lysates were prepared for western blotting. (g) Extension of TGF-β-induced Smad phosphorylation in HaCaT cells carrying TMEPAI-specific shRNA. HaCaT cells introduced with either non-targeting or TMEPAI-specific shRNA#5 were

stimulated with 0.5 ng/ml TGF-β for indicated times. The expressions for C-terminal phosphorylation of Smad2 and Smad3 were normalized using the intensity of the band corresponding to Smad2 and Smad3, respectively. Inducibility was calculated relative to the value for cells in the absence of TGF-β. (h) Reduced expression of TMEPAI in cells enhances expression of JunB upon TGF-β stimulation. HaCaT cells were infected with lentiviruses expressing TMEPAI-specific shRNAs (#2 and #5). Cells were stimulated with 5 ng/ml TGF-β for 10 h. Three hours before lysis of cells, Tran[^{35}S]-Label was added to the medium. Subsequently, immunopreicipitation with anti-JunB antibody (SantaCruz) was carried out. The expression of JunB was normalized using the intensity of the band corresponding to β-actin. Inducibility was calculated relative to the value for non-targeting shRNA-infected cells in the absence of TGF-β.

Fig. 2. Interaction of TMEPAI with R-Smads. (a) Interaction of TMEPAI with Smads. COS7 cells were transfected with indicated plasmids and harvested for co-immunoprecipitation (Co-IP) experiments. (b) Interaction of TMEPAI with Smad2 *in vitro*. *In vitro* translated TMEPAIΔTM was incubated with GST alone, GST-Smad2 or GST-Smad2(W368A). [^{35}S]-labeled TMEPAIΔTM bound to GST fusion protein was precipitated using GSH-Sepharose4B (GE Healthcare). After loading precipitates including GSH-Sepharose4B to SDS-PAGE, labeled proteins were detected using BAS2500 (Fuji film) (upper panel). Simultaneously, the gel was stained with Coomassie Brilliant Blue R-250 (CBB) to show GST proteins (lower panel). (c) TMEPAI equally binds to non-phosphorylated and phosphorylated Smad2. *Left panel*, illustration of how cell lysates were prepared from each dish in which indicated plasmids were transfected.

Right panel, each cell lysate was mixed and subjected to Co-IP experiments. (d) TMEPAI interferes with the Smad2-Smad4 complex formation. COS7 cells were transfected with indicated plasmids and harvested for Co-IP experiments. (e) Endogenous interaction of TMEPAI with non-phosphorylated and phosphorylated Smad2 after TGF-β stimulation. HaCaT cells were stimulated with 5 ng/ml TGF-β for 8 h and harvested for Co-IP experiments. Immunoprecipitation was carried out using a mouse anti-TMEPAI monoclonal antibody (Abnova). The membrane was blotted with an anti-Smad2, an anti-phosphorylated Smad2 (PS2) or an anti-TMEPAI polyclonal antibody. As a negative control, mouse IgG was used for immunoprecipitation. An asterisk indicates the band corresponding to C-terminal phosphorylated Smad2.

Fig. 3. Determination of functional domain in TMEPAI. (a) Schematic presentation of deletion mutants for mouse TMEPAI. TM; transmembrane domain, PY; PY motif. (b) Interaction of TMEPAI mutants with Smad2. COS7 cells were transfected with indicated plasmids and harvested for Co-IP experiments. (c) Effect of TMEPAI mutants on TGF-β-induced reporter activity. Different doses of TMEPAI or its mutants were co-transfected with $(CAGA)_{12}$-luc in HepG2 cells with or without 5 ng/ml TGF-β for 18 h. (d) Effect of TMEPAI mutants on ALK5ca-induced Smad2 phosphorylation. Experiments were performed according to Fig. 1b. (e) Schematic presentation of TMEPAI(4A) mutant. (f) Interaction of TMEPAI(4A) mutant with Smad2. COS7 cells were transfected with indicated plasmids and harvested for Co-IP experiments. (g) Effect of TMEPAI(4A) mutant on ALK5ca-induced Smad2 phosphorylation. Experiments were performed according to Fig. 1b. (h) TMEPAI(4A) does not inhibit TGF-β-induced reporter activity.

Experiments were performed according to Fig. 3c. (i, j) TMEPAI, but not TMEPAI(4A), blocks nuclear translocation of Smad2 upon TGF-β stimulation. NMuMG cells were transfected with (i) TMEPAI or (j) TMEPAI(4A), stimulated with 5 ng/ml TGF-β for 1 h and fixed for immunofluorescence. Ectopic TMEPAI and endogenous Smad2 were visualized with red and green, respectively. White arrows indicate TMEPAI-expressing cells in which Smad2 did not translocate into the nucleus upon TGF-β stimulation. Yellow arrows indicate TMEPAI(4A)-expressing cells in which Smad2 could translocate into the nucleus upon TGF-β stimulation.

Fig. 4. TMEPAI co-localizes with SARA in endosomes and competes with SARA for binding to Smad2. (a) Substitution of Trp^{368} to Ala in Smad2 abrogates the ability of Smad2 to interact with TMEPAI. COS7 cells were transfected with indicated plasmids and harvested for Co-IP experiments. (b, c) SARA(SBD) and TMEPAI prevent interaction of Smad2 with (b) TMEPAI and (c) SARA(SBD), respectively. Each cell lysate was mixed and subjected to Co-IP experiments according to Fig. 2c. (d) GFP-SARA(FYVE) and TMEPAI/V5 were co-transfected in 911 cells. Twenty-four hours after transfection, cells were fixed and stained with mouse anti-V5 monoclonal antibody. Then, TexRed-conjugated goat anti-mouse IgG antibody (Molecular Probe) was used for visualization. (e) Electron microscopy of endosome-like structure containing GFP-FYVE and TMEPAI/V5. MCF10A1 cells were transfected with GFP-FYVE and TMEPAI/V5. After the fixation of cells, immunostaining was performed with (small dots) mouse monoclonal anti-V5 antibody (10 nm-gold) and (large dots) rabbit polyclonal anti-GFP antibody (15 nm-gold). Arrows point to TMEPAI-linked 10 nm-gold particles. (f) SARA

rescues TGF-β-induced transcription perturbed by TMEPAI. TMEPAI and $(CAGA)_{12}$-luc were co-transfected with different doses of SARA in HepG2 cells with or without 5 ng/ml TGF-β for 18 h. (g) Recuperative effect of SARA on ALK5ca-induced Smad2 phosphorylation blocked by TMEPAI. Experiments were performed according to Fig. 1b.

Fig. 5 TMEPAI blocks activin signaling in *Xenopus* embryos. (a) Ectopic expression of xTMEPAI in dorsal cells. mRNA coding for xTMEPAI (50 pg) was injected into two dorsal blastomeres at the 4-cell stage. Phenotypes of embryos were determined at the tadpole stage (the typical examples are shown). (b) Inhibitory effect of xTMEPAI on expression of mesoderm markers in animal caps stimulated with activin. xTMEPAI mRNA was injected alone or together with activin mRNA (2 pg) near the animal pole of 2-cell embryos and animal caps were explanted at the blastula stage. Total RNAs were prepared and analyzed by RT-PCR. Histone was used as an internal control. +RT and –RT indicate reverse transcriptional reaction using normal embryos with and without reverse transcriptase, respectively. (c) RT-PCR analysis of the DMZ expressing xTMEPAI. xTMEPAI mRNA was injected into the DMZ at the 4-cell stage. Subsequently, RNAs were prepared from embryos at stage 11. +RT and –RT indicate reverse transcriptional reaction using normal embryos with or without reverse transcriptase. (d) Knockdown of xTMEPAI enhances expression of mesoderm markers in animal caps stimulated with activin. TMEPAI MO and control MO were injected alone or together with activin mRNA (3 pg) near the animal pole of 2-cell embryos and animal caps were explanted at the blastula stage. All other experiments were performed according to Fig. 5b.

Fig. 6 A model of TMEPAI regulatory action on TGF-β signaling. After ligand-induced heteromeric TGF-β receptor complex formation, R-Smad (*i. e.* Smad2 and Smad3) are recruited by SARA (via its SBD) to the active TGF-β type I receptor upon which they get phosphorylated. Activated R-Smads form the ternary complex with Smad4, which translocate to the nucleus where they bind to promoters of target genes including TMEPAI together with transcription factor(s) (TF) and co-activator(s), and activate transcription of target genes. Then, various direct target molecules for TGF-β/Smad signaling are induced. TMEPAI (via its SIM domain) competes with SARA for interaction with R-Smads. Thus, TMEPAI sequesters R-Smads from SARA and perhaps other (scaffolding) proteins. Besides, TMEPAI captures phosphorylated Smad2 and Smad3 to prevent their nuclear translocation. Thereby, TMEPAI regulates the duration and intensity of TGF-β responses. NTD; amino-terminal domain, CTD; carboxy-terminal domain.