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

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Abstract

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 (SIM). TMEPAI competes with Smad anchor for receptor activation (SARA) for R-Smad binding, thereby sequestering R-Smads from TβRI kinase activation. In mammalian cells, ectopic expression of TMEPAI inhibited TGF-β-induced PAI-1 production, whereas specific siRNA-mediated knockdown of TMEPAI expression potentiated TGF-β-induced Smad2 phosphorylation and cellular responsiveness by TGF-β. Consistently, TMEPAI inhibits activin-mediated mesoderm formation in *Xenopus* embryos. Taken together, TMEPAI participates in a negative feedback loop to control the duration and intensity of TGF-β 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 (1). 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) is phosphorylated and activated by TβRII. Active TβRI catalyzes the C-terminal serine phosphorylation of receptor-regulated (R-)Smad proteins. 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 (2).

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 (3). Because they operate as morphogens, inducing distinct cell fates at different ligand concentrations, duration and intensity are critical determinants in specifying 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, the Smad anchor for receptor activation (SARA) has been shown to recruit non-activated Smads to the activated TGF-β receptor complex (4). However, it is not known whether molecules

exist that antagonize this function. 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 (1,5).

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 (6-8). The transcript of TMEPAI was recently shown to be induced by TGF-β (9-11). 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 (12) and cell growth inhibition (10). However, the mechanism of its action and physiological function is 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. We first examined if TMEPAI is indeed a direct target gene of TGF-β signaling. TGF-β potently stimulated TMEPAI expression in AML cells (Fig. 1a). To explore if TMEPAI is a direct target gene of TGF-β signaling, the cells were treated for 2 h with cycloheximide (CHX) 1 h prior to TGF-β stimulation. As seen in Fig. 1b, 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. Thus, TMEPAI is an immediate-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 (Fig. 1c). Challenging C2C12 cells with BMP could not induce the expression of TMEPAI (Supplementary Fig. 1a), unlike I-Smads whose transcripts are known to be elevated by multiple TGF-β family members (13,14).

Expression of TMEPAI is increased with tumorigenicity. Since TMEPAI is reported to be highly expressed in breast cancer (7), we checked the expression of TMEPAI in established cells used as breast cancer models at different stages of carcinogenesis (15). Among the cell lines examined, expression of TMEPAI was lowest in MCF10A1, characterized as a model of normal breast epithelium, whilst TMEPAI was expressed at the highest level in MCF10CA1h, characterized as a model of low-grade breast carcinoma. MCF10AT1, categorized as a premalignant epithelium, showed intermediate TMEPAI expression (Fig. 1d). In contrast, both phosphorylation of R-Smad by TGF-β and production of fibronectin by TGF-β were decreased with malignancy of breast cancer cells (15 and data not shown). A recent report demonstrated that high expression of TMEPAI was observed in adenomas from *Apc*$^{min/+}$ mice, which were used extensively as a mouse model of the human colorectal cancer syndrome, familial adenomatous polyposis coli (16). Immunohistochemistry on consecutive sections from *Apc*$^{min/+}$ mouse adenoma revealed that the expression of TMEPAI is seen in Ki-67-positive tumor cells where β-catenin is prominently expressed in the nucleus, but not in surrounding stroma cells (Fig. 1e). Thus, it is possible that expression of TMEPAI enhances malignancy in tumor cells by abrogating the cytostatic action of TGF-β.

TMEPAI perturbs TGF-β signaling. I-Smads, Smurfs, SnoN, and TGIF involved in a negative feedback loop that controls TGF-β responses are immediate-early response genes for TGF-β signaling (1,5). To investigate if TMEPAI modulates cellular responsiveness to TGF-β, we tested the effect of TMEPAI on the Smad-driven transcriptional $(SBE)_4$-luc reporter (17). 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. 2a). Since TMEPAI is a type Ib transmembrane protein that might be localized to plasma membrane, TMEPAI might exert its negative role on TGF-β/Smad signaling by interfering with R-Smad phosphorylation. To test 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 (18). TMEPAI markedly reduced phosphorylation of both Smad2 and Smad3 upon ALK5 activation (Fig. 2b and c). Similarly, TMEPAI also blocked activin type I receptor (ALK4)-induced Smad2 phosphorylation (Fig. 2d). On the other hand, BMP type I receptor (ALK6)-induced Smad1 phosphorylation was not influenced by TMEPAI (Supplementary Fig. 1b). Three variants of the human TMEPAI counterpart have been reported (Supplementary Fig. 2a). Two possess the transmembrane domain (TM), whereas the third is deficient of TM. Like mouse TMEPAI, all of the human variants could block TGF-β-induced reporter activity and Smad2 phosphorylation (Supplementary Fig. 2b and c). Thus, all of the human TMEPAI isoforms can inhibit TGF-β signaling despite the inhibitory ability of each isoform being unequal. In addition, there are 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 cells from producing PAI-1, a TGF-β direct target gene, upon TGF-β stimulation, whereas TMEPAI(4A), a non-functional TMEPAI mutant (see Fig. 4e-h), did not affect production of PAI-1 by TGF-β (Fig. 2e). Conversely, siRNAs corresponding to mouse TMEPAI were introduced into NMuMG cells, and ALK5ca-induced PAI-1 expression was then measured. As expected of a negative role of TMEPAI in TGF-β signaling, PAI-1 produced by NMuMG cells upon ALK5 activation was further enhanced after treatment of cells with TMEPAI-specific siRNA (Fig. 2f). Consistent with the increase in ALK5ca-induced PAI-1 production using TMEPAI siRNA, TMEPAI-specific knockdown augmented ALK5ca-mediated Smad2 phosphorylation (Fig. 2g,h). These results indicate that TMEPAI is a physiologically critical molecule for attenuating 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 (13). Compared with Smad7, TMEPAI only marginally interacts with the TGF-β receptor complex (Supplementary Fig. 3a), 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. 3a 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. However, we were unable to distinguish phosphorylated R-Smads from non-phosphorylated R-Smads in this experiment because TMEPAI blocks R-Smad phosphorylation (Fig. 2b and c). Therefore, 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 and then immunoprecipitated with anti-Flag antibody and analyzed by Western blotting with anti-V5 antibody. As seen in Figure 3b, 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 was detected (Supplementary Fig. 3b).

After TGF-β stimulation, phosphorylated Smad2 can form a heteromeric complex with Smad4 and then enter the nucleus to regulate target gene expression (1). Since TMEPAI blocks TGF-β-induced Smad2 phosphorylation, we speculated that TMEPAI may perturb the Smad2/Smad4 interaction upon receptor activation. Indeed, ectopic expression of TMEPAI was found to inhibit complex formation between Smad2 and Smad4 upon ALK5 activation (Fig. 3c). To show the physiological significance of the association between TMEPAI and Smad2, we investigated the endogenous interaction of TMEPAI with Smad2 in NMuMG cells. As TMEPAI expression in the absence of TGF-β is low, the cells were treated with TGF-β for 8 h (Fig. 1c). As expected, TMEPAI interacted with Smad2 in non-transfected NMuMG cells (Fig. 3d). The latter interaction is specific as TMEPAI could not interact with the closely related Smad1 (Supplementary Fig. 1c). Taken together, we concluded that TMEPAI induced by TGF-β preferentially

associates with TGF-β/activin R-Smads (ie, Smad2 and Smad3) to inhibit ALK5-mediated R-Smad phosphorylation.

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 (19). However, all of the TMEPAI mutants lacking the PY motifs (TMEPAIΔPY1, TMEPAIΔPY2, and TMEPAIΔPY) retained their inhibitory ability of TGF-β signaling (Supplementary Fig. 4a-c). Like human TMEPAI isoform C (Supplementary Fig. 2b and c), TMEPAI lacking its transmembrane domain (TMEPAIΔTM) (Supplementary Fig. 4a), which is mislocalized in cytosols (data not shown), could also inhibit, albeit more weakly, the TGF-β-induced reporter activity (Supplementary Fig. 4b). 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. 4a) and tested for their ability to interact with Smad2. The domain from ^{171}Asn to ^{204}Ser is necessary for its interaction with Smad2 (Fig. 4b). Consistent with the interaction of TMEPAI mutants with Smad2, TMEPAI(1-171) lacking the Smad2-binding domain had no ability to block TGF-β receptor-induced responses (Fig. 4c and d).

When we carefully checked the domain from ^{171}Asn to ^{204}Ser in TMEPAI, we found the peptide sequence (Pro-Pro Asn-Arg; PPNR) similar to the Smad interaction motif (SIM) that was originally discovered in the transcriptional factors Milk and Mixer (20). To examine the significance of the PPNR sequence in TMEPAI in its inhibitory

action of TGF-β signaling, we replaced ^{178}PPN^{181}R in TMEPAI with AAAA and termed it TMEPAI(4A) mutant (Fig. 4e). When TMEPAI(4A) was overexpressed in COS7 cells together with Smad2, TMEPAI(4A) no longer interacted with Smad2 (Fig. 4f). Consistently, TMEPAI(4A) could not block TGF-β-induced Smad2 phosphorylation (Fig. 4g) and reporter activity (Fig. 4h). These results indicate the critical function of the SIM in TMEPAI in inhibiting TGF-β signaling. If TMEPAI tightly interacts with Smad2, expression of TMEPAI in cells should antagonize Smad2 nuclear translocation after treatment of the cells with TGF-β. To confirm this possibility, NMuMG cells transiently expressing TMEPAI were stimulated with TGF-β for 1 h. As shown in Fig. 4i, TGF-β-induced Smad2 nuclear accumulation was blocked upon ectopic TMEPAI expression. On the other hand, TMEPAI(4A), defective in Smad2 binding, did not affect TGF-β-induced Smad2 nuclear accumulation (Fig. 4j).

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. 5). Previously it was reported that ^{368}Trp in the MH2 domain of Smad2 is critical for Smad2 to bind to the SIM domain of Milk and Mixer (20). Therefore, we explored whether Trp^{368} in Smad2 is necessary for the interaction with TMEPAI. As seen in Fig. 5a, Smad2(W368A) mutant had no ability to interact with TMEPAI. The tryptophan residue critical for TMEPAI interaction is conserved in Smad2 and Smad3, but not in other Smads. This provides an explanation for 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 (4). The amino acid sequence of the rigid coil in SARA SBD is similar to that of the SIMs in Milk and Mixer (20), but the flanking amino acid sequence other than PPNR in TMEPAI has no similarity to SARA SBD. Ectopic expression of SARA(SBD) (21), however, abrogated the interaction between TMEPAI and Smad2 (Fig. 5b). In addition, TMEPAI could disrupt the interaction between Smad2 and SARA(SBD) (Fig. 5c). SARA has been reported to require ^{368}Trp and ^{381}Asn residues in Smad2 for its efficient binding to Smad2 (22). Indeed, Smad2(W368A) still has the ability to interact with SARA (data not shown). Occupation of Trp^{368} in Smad2 by TMEPAI possibly affects SARA-Smad2 complex formation, and therefore SARA SBD might not reach Asn^{381} in Smad2. These results indicate that TMEPAI interferes with the SARA-Smad2 complex. 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 shares 70% and 66% amino acid sequence identity with human and mouse TMEPAI, respectively (Supplementary Fig. 6). However, the TM, SIM and PY motifs in TMEPAI of the 3 species are almost identical. We analyzed patterning phenotypes caused by overexpression of *Xenopus* TMEPAI (xTMEPAI) in *Xenopus* embryos. When the endogenous activin signaling pathway is inactivated in early *Xenopus* embryos, mesoderm fails to form (23,24).

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. 6a). 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 (25). 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. 6b). 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. 6c). 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. As expected, the injection of xTMEPAI morpholino oligonucleotide (TMEPAI MO) augmented expression of Xbra and gsc in comparison with that of the control MO (scramble MO) or the mock-injected (Fig. 6d). 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 (1). Disruption of the balance between positive and negative regulation in TGF-β signaling can lead to various diseases (3). We have herein presented evidence demonstrating that TMEPAI, a transmembrane protein, is implicated 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. As expected, TMEPAI negatively controls the TGF-β signaling pathway. An interesting question is why TMEPAI appears to be responsible for inhibition of TGF-β or activin signaling, but not that of BMP signaling. Although we initially thought that the PY motifs in TMEPAI are 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. 4a-c). Indeed, the SIM domain in TMEPAI (PPNR) was a critical domain in preventing TGF-β signaling. As in a previously described experiment that used the SIM domain in Mixer (26), the SIM domain in TMEPAI also showed almost the same 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 terminate TGF-β signaling efficiently. Binding of Smad2 and Smad3 to TMEPAI is mutually exclusive with binding to SARA. Consequently, TMEPAI sequesters Smad2 and Smad3 from SARA so that it cannot recruit Smad2 and Smad3 to the activated TGF-β receptor complex (Fig. 7). 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 (27). However, Hgs does not have a SIM domain in

its structure, which demonstrates that TGF-β signaling through Hgs might be insensitive to TMEPAI. 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 interaction between Milk or Mixer and SARA remains veiled because Milk and Mixer are nuclear proteins (20). Our present results elucidated a physiological significance of the SIM domain 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 (28). 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 of *Xenopus* embryos could confirm the same specific inhibitory action of TMEPAI on the TGF-β/activin pathway through Smad2 and Smad3 as that in the mammalian system. It is possible that TMEPAI makes a gradient in *Xenopus* embryos from the ventral to the dorsal region for developmental fine-tuning.

Escape from TGF-β-mediated anti-proliferative effect leads normal cells to acquire tumorigenicity. An experiment using breast cancer models at different stages of tumorigenicity revealed that expression of TMEPAI increases with malignancy. Indeed, the expression of TMEPAI is relatively high in the tumorigenic region of patients with breast cancer (7). 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 (29). Thus, temporal interruption of TGF-β signaling by overexpression of TMEPAI might be a desirable process for tumor cells. *Apc*$^{min/+}$ mice develop multiple intestinal adenoma in which biallelic inactivating mutation of the *Apc* gene occurs (30,31). Our immunohistochemistry 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). 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 (32). 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. Besides, it must be important to investigate 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 novel type of TGF-β/activin-specific blocker 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.

Materials and Methods

Expression Plasmids – TMEPAI cDNA for mouse, human and *Xenopus laevis* were cloned by the RT-PCR method. All of the mouse TMEPAI mutants and Flag-Smad2(W368A) were made with a QuickChange site-directed mutagenesis kit (Stratagene) or pfx polymerase (Invitrogen). TMEPAI and its mutants other than *Xenopus* TMEPAI were inserted into pcDNA3.1-V5-His-A (Invitrogen) or pcDNA3-HA (33), whilst *Xenopus* TMEPAI tagged with Flag epitope was inserted into pCS2. All TMEPAI constructs possess Flag-, HA- or V5-epitope tags at their C-terminus. GFP-SARA(SBD) was constructed by introduction of the SBD in SARA (^{666}Ser to ^{722}Glu) into pEGFP-C3 (Clontech). V5-Smad2 was constructed by substitution of Flag epitope with V5 epitope in Flag-Smad2 (34). The construction methods of ALK5ca/V5, ALK5ca/HA, Flag-Smad2, 6xmyc-Smad2, Flag-Smad3, Flag-Smad4, Flag-Smad7, $(SBE)_4$-luc, and $(CAGA)_{12}$-luc were described previously (17, 34-36). Adenoviruses expressing ALK5ca/HA and LacZ were previously mentioned (37).

Cell Culture – HepG2, NMuMG, NIH3T3, AML, and COS7 cells were cultured in Dulbecco's modified Eagle's medium (Sigma) containing 10% fetal calf serum (Bio-west) and 1x MEM non-essential amino acids (Invitrogen). MCF10A1 and its derivatives were maintained by the method described previously except for use of 5 μM forskolin instead of cholera toxin (15).

Immunohistochemistry –The paraffin-embedded tissues were sectioned to a 3 μm thickness, deparaffinized in xylene, rehydrated in graded ethanol solution, and immersed in citrate-NaOH buffer (10 mM sodium citrate, pH 7.2) for 20 min at 115˚C to restore antigenicity. The rehydrated sections were stained with hematoxylin and eosin, or

incubated overnight at 4˚C with mouse anti-α-TMEPAI antibody (Abnova, 1:100), rabbit anti-Ki67 antibody (Ylem S.R.L., 1:100), or mouse anti-β-catenin antibody (BD Transduction laboratories, 1:100). The sections incubated with the first antibodies were washed with PBS and subsequently colored using the Dako EnVision+system HRP (DAB) (DakoCytomation).

RT-PCR, RNA preparation, transfection, reporter assay, immunoprecipitation, Western blotting and immunofluoresence– RT-PCR, RNA preparation, transient transfection, reporter assay, immunoprecipitation, Western blotting, and immunofluoresence were performed as previously described (34,36,37). In all reporter assays, β-galactosidase expression vector pCH110 (Pharmacia) 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 synthesizing peptide (LSDGEEPPPYQGPC).

PAI-1 production– NMuMG cells were seeded in a 1 x 10^6/6-well plate one day before transfection. The cells were first transfected with TMEPAI-specific siRNA mixture purchased from Dharmacon according to the attached instruction manual and then infected with ALK5ca/HA or LacZ adenoviruses. After 12 h of infection, the cells were metabolically labeled with Tran[^{35}S]-Label (0.37 MBq/ml; MP Biochemicals) for 3 h. Subsequently, ^{35}S-labeled PAI-1 produced by the cells was prepared as described previously (36).

[^{32}P]Orthophosphate labeling of cells– NIH3T3 cells were transfected with siRNAs for 24 h and then infected with adenoviral ALK5ca. Two hours before lysis, [^{32}P]orthophosphate (37 MBq/ml; MP Biochemicals) was added to the medium. The cell

lysates were immunoprecipitated with anti-Smad2 antibody (BD Transduction laboratories) to show phosphorylated Smad2.

Microinjection of synthetic mRNA and RT-PCR analysis for Xenopus embryos– Unfertilized eggs were collected and fertilized *in vitro* as previously described (38). The embryos were subsequently dejellied using 3% cysteine. Capped mRNAs for each protein to be tested were synthesized from linearized constructs using the mMESSAGE mMACHINE SP6 kit (Ambion). For animal cap assays, mRNAs were injected into the animal poles of 2-cell-stage embryos. Injected embryos were kept in 3% Ficoll/0.1 x Steinberg's solution as previously described (39). Animal cap explants were dissected with hair knives at stages 8 to 9. For ventral induction, synthetic mRNAs were injected into two dorsal blastomeres at the 4-cell stage. Embryos were examined for phenotype at the tadpole stage. Animal caps were collected from sibling embryos at stage 12, and total RNA was extracted using TRIzol (Gibco/BRL) according to the manufacturer's instructions. cDNA was synthesized from extracted RNAs as previously described (39) for RT-PCR analysis. PCR was performed with the oligonucleotide primer pairs: Xbra, 5'-TCCCAATGCAATGTATACGGTTCTG-3' (upstream) and 5'-ATTCTGGTATGCGGTCACTGCTATG-3' (downstream); gsc, 5'-ACTACTATGGACAGTTGCACG-3' (upstream) and 5'-TTCTGATTCCTCTGATGAAGATC-3’ (downstream). The primer sequence of histone H4, an internal input control, was previously described (40). The sequence of the morpholino oligonucleotide for *Xenopus* TMEPAI is 5’-ATAGTCTGGAACAGAGACCTCTTGC-3’.

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Figure legends

Fig. 1. TMEPAI is a direct target gene for TGF-β signaling. (a) Induction of TMEPAI mRNA by TGF-β. AML cells were treated with 5 ng/ml TGF-β for indicated times. Then, RT-PCR was performed using total RNAs. (b) *De novo* protein synthesis is not required for induction of the TMEPAI transcript by TGF-β. AML cells were stimulated with 5 ng/ml TGF-β 2 h after pretreatment with 5 ng/ml CHX for 1 h. RT-PCR was carried out according to Fig. 1a. (c) Expression of TMEPAI protein after treatment with TGF-β. NMuMG cells were treated with 5 ng/ml TGF-β for indicated times and then analyzed by Western blotting with anti-TMEPAI antibody. (d) Expression of TMEPAI in breast cancer cell lines representing different stages in tumor progression. Cell lysates were prepared from MCF10A1 as a normal epithelium, MCF10AT1 as a premalignant epithelium, and MCF10CA1h as a low-grade carcinoma, and then analyzed by Western blotting with anti-TMEPAI antibody. (e) Paraffin-embedded sections of intestinal adenoma from $Apc^{min/+}$ mice. Adenoma was stained for (i) β-catenin, (ii) TMEPAI and (iii) Ki-67 using 3 consecutive sections.

Fig. 2. 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-Smad3 antibody (PS1). (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 anti-phospho-Smad2 antibody (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, TMEPAI- or TMEPAI(4A)-expressing adenoviruses. Cells were treated with TGF-β for 6 h. Three hours before lysis of cells, Tran[^{35}S]-Label was added to the medium. (f) Reduced expression of TMEPAI in cells enhances PAI-1 production. Cells were transfected with siRNAs for 24 h and then infected with adenoviral ALK5ca. Three hours before lysis of cells, Tran[^{35}S]-Label was added to the medium. PAI-1 production was normalized using the intensity of the band corresponding to β-actin. Inducibility was calculated relative to the value for controlling siRNA-treated cells in the absence of ALK5. (g) Specific TMEPAI knockdown in NIH3T3 cells. Cells were infected with either ALK5ca- or LacZ-expressing adenovirus. (h) Phosphorylation of Smad2 upon ALK5 activation is potentiated by treatment of NIH3T3 cells with TMEPAI-specific siRNAs. NIH3T3 cells were transfected with siRNAs for 24 h, and then infected with adenoviral ALK5ca. Two hours before lysis, [^{32}P]orthophosphate was added to the medium.

Fig. 3 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) 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. (c) TMEPAI interferes with the Smad2-Smad4 complex. COS7 cells were transfected with indicated plasmids and harvested for Co-IP experiments. (d) Endogenous interaction of TMEPAI with Smad2 after TGF-β stimulation. NMuMG cells were stimulated with 5 ng/ml TGF-β for 8 h and harvested for Co-IP experiments.

Fig. 4. Determination of functional domain in TMEPAI. (a) Schematic presentation of deletion mutants for 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 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. 2b. (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. 2b. (h) TMEPAI(4A) does not inhibit TGF-β-induced reporter activity. Experiments were performed according to Fig. 4c. (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 immunofluoresence.

Ectopic TMEPAI and endogenous Smad2 were visualized with green and red, respectively. Arrows reveal TMEPAI-expressing cells in which Smad2 could not translocate into the nucleus upon TGF-β stimulation.

Fig. 5. TMEPAI competes with SARA for binding to Smad2. (a) Substitution of ^{368}Trp to Ala in Smad2 leads Smad2 to lose the ability 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. 3b.

Fig. 6 TMEPAI blocks activin signaling in *Xenopus* embryos. (a) Overexpression 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 were 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. Cells are normally fated to express mesoderm markers;

however, xTMEPAI blocks the expression of those markers. +RT and –RT indicate reverse transcriptional reaction using normal embryos with or without reverse transcriptase. (d) Knock-down 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. 6b.

Fig. 7 **A model of TMEPAI action on TGF-β signaling.** After the ligand-receptor complex, recruitment of R-Smad (*i. e.* Smad2 and Smad3) to active type I receptor by SBD in SARA is followed by phosphorylation of R-Smads by active type I receptor kinase. Then, various direct target genes of TGF-β/Smad signaling are induced. Among them, TMEPAI competes with SARA to trap R-Smads via its SIM domain. Thus, TGF-β/Smad signaling is terminated because SARA can no longer present R-Smads to the active type I receptor. NTD; amino-terminal domain, CTD; carboxy-terminal domain.

Fig. 1 (Watanabe et al.)

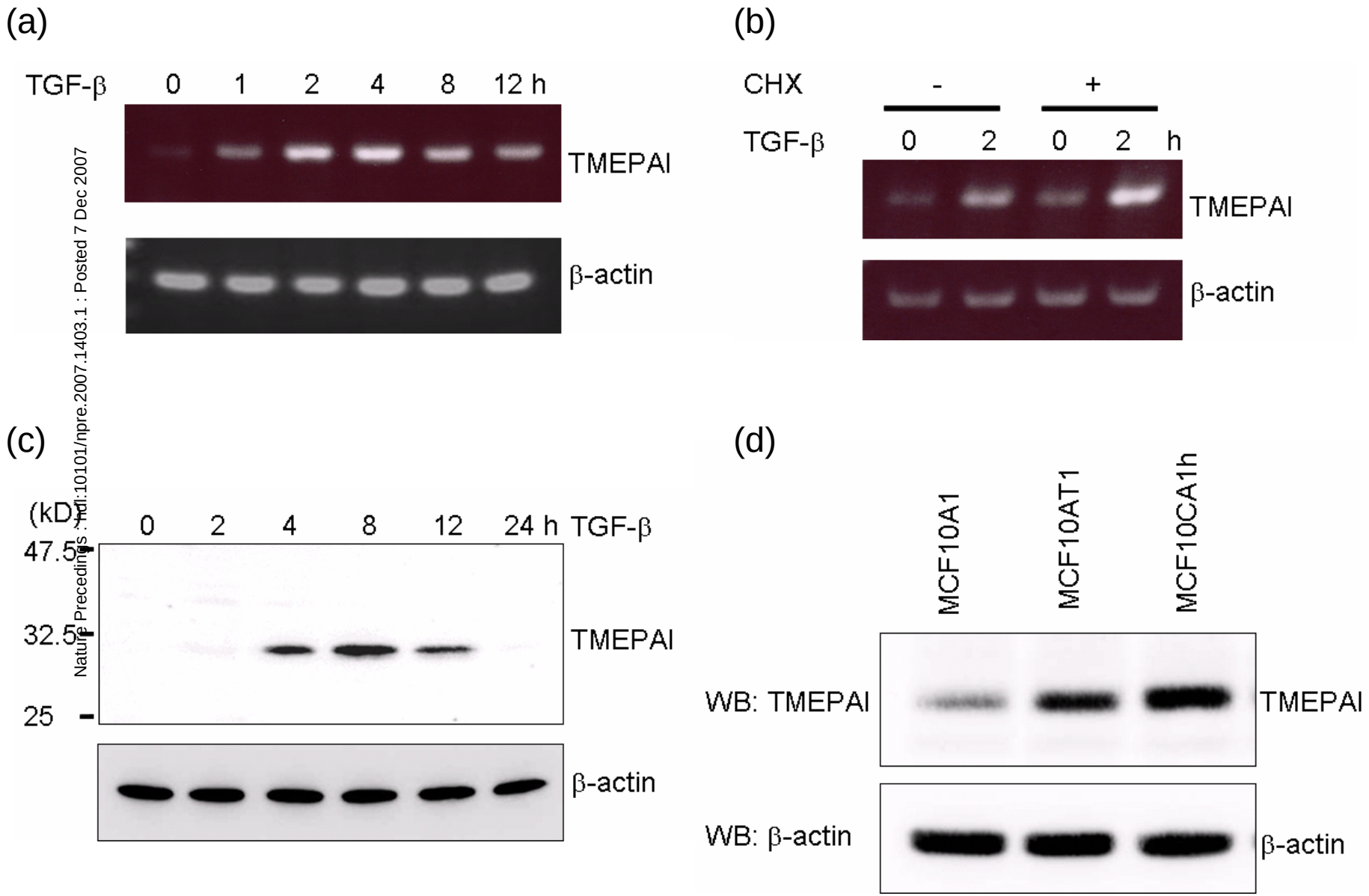
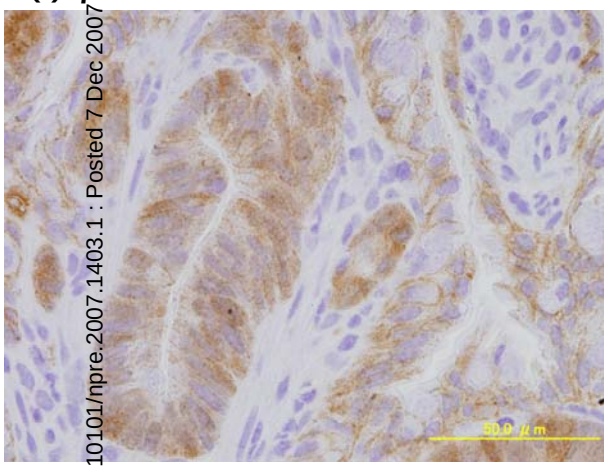


Fig. 1 (Watanabe et al.)

(e)

(i) β-catenin



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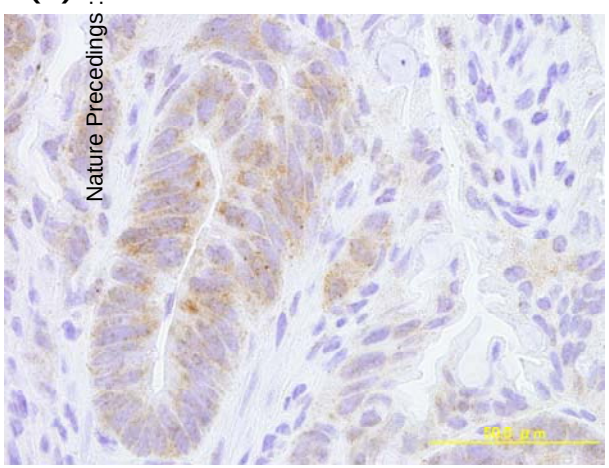


(iii) Ki-67

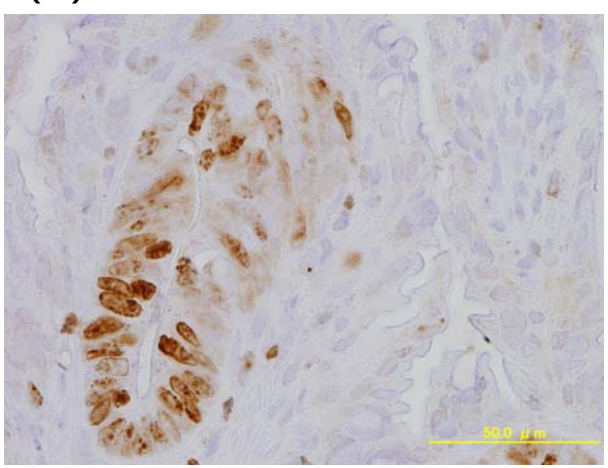
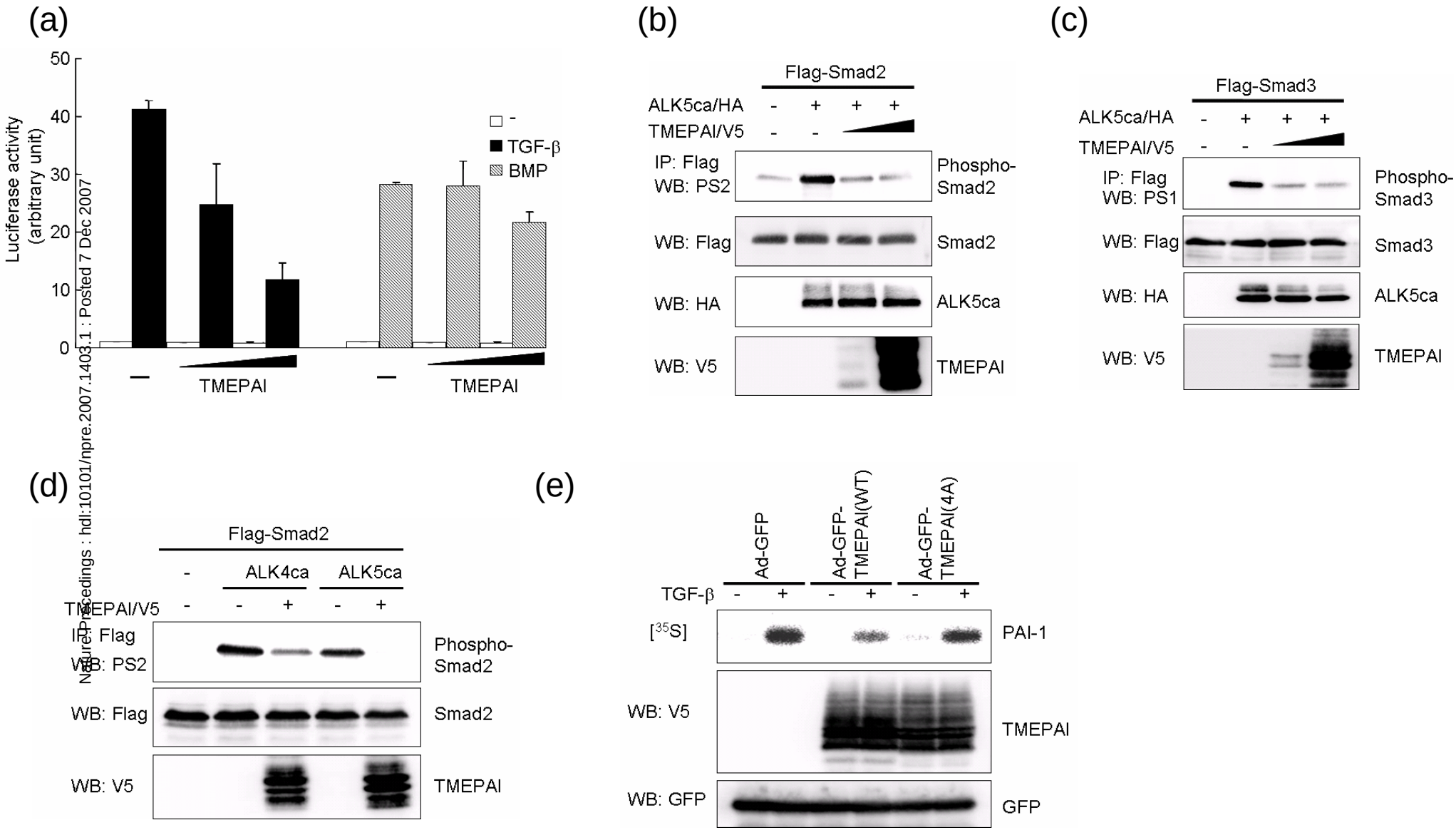


Fig. 2 (Watanabe et al.)



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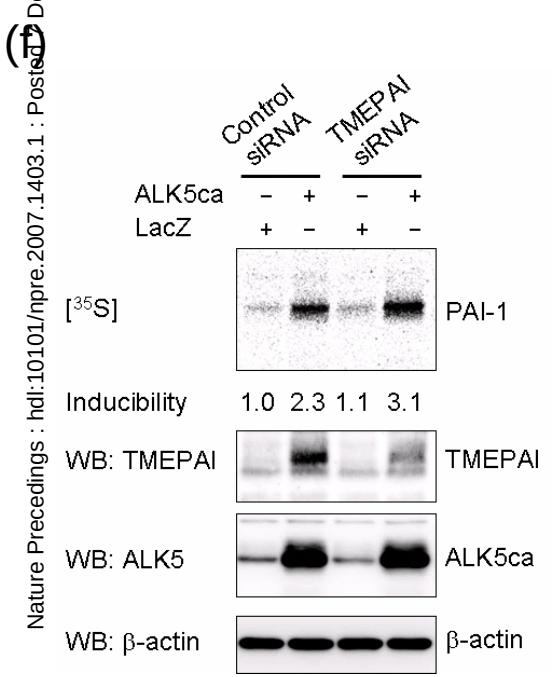

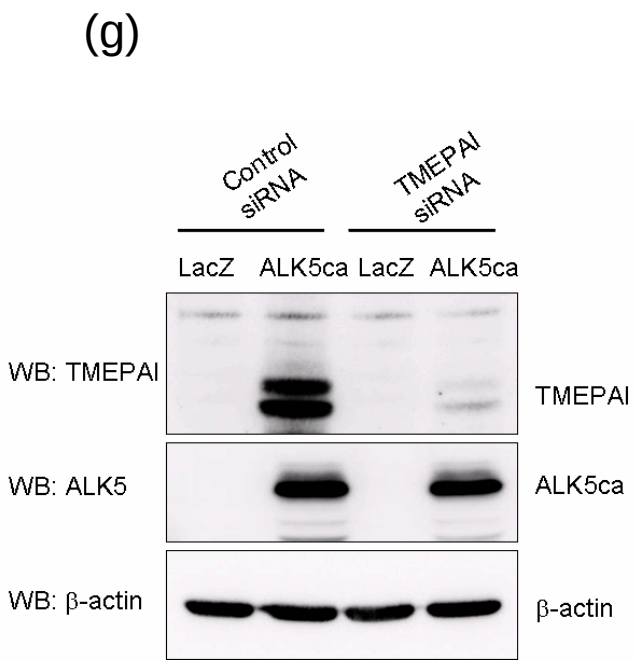

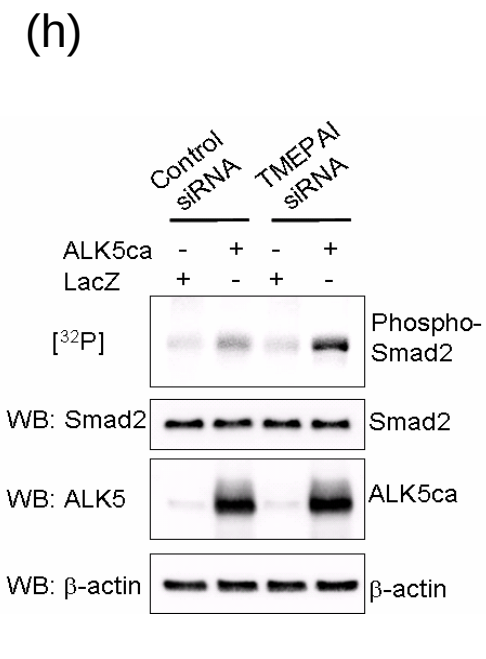

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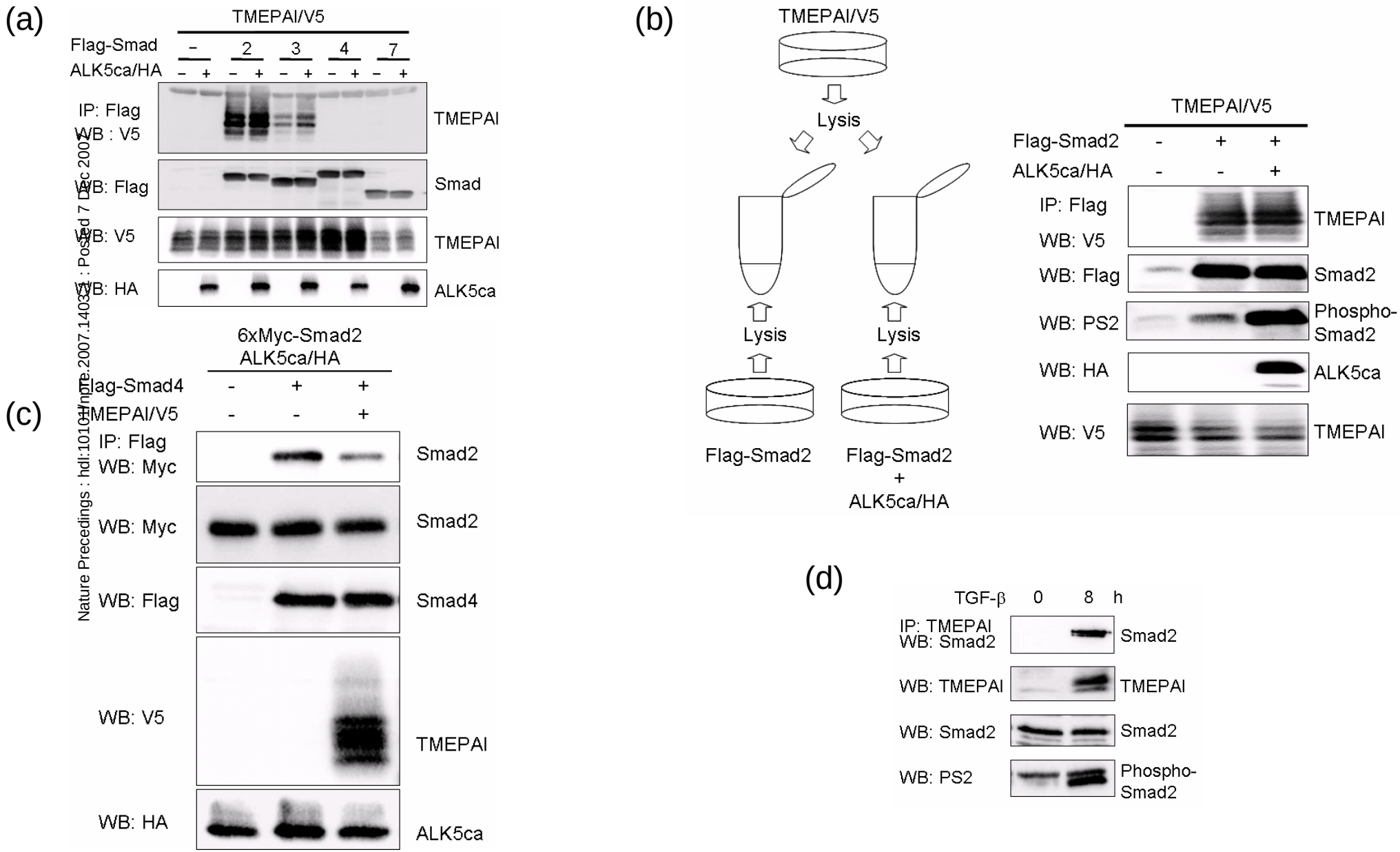

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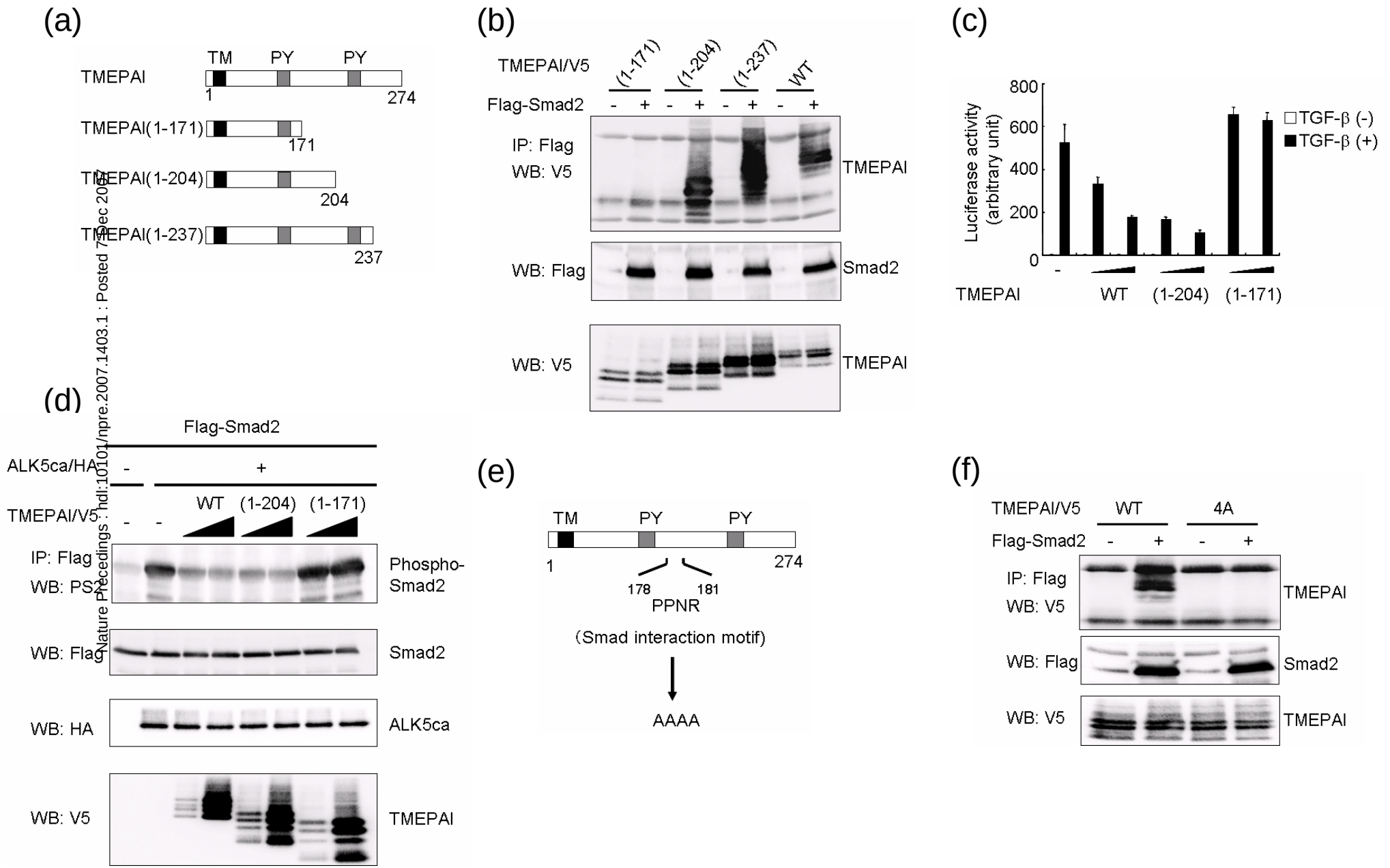
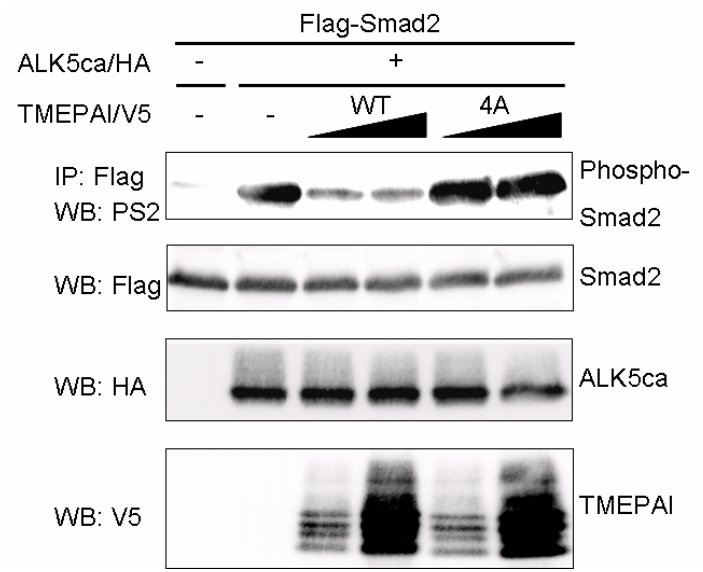


Fig. 4 (Watanabe et al.)

(g)



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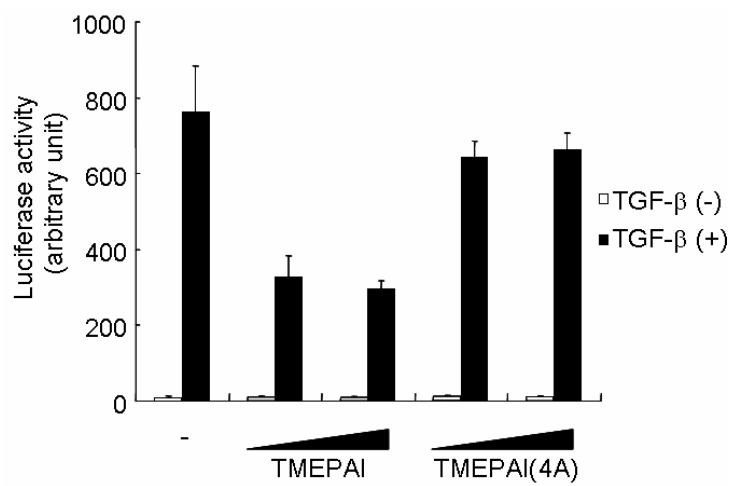

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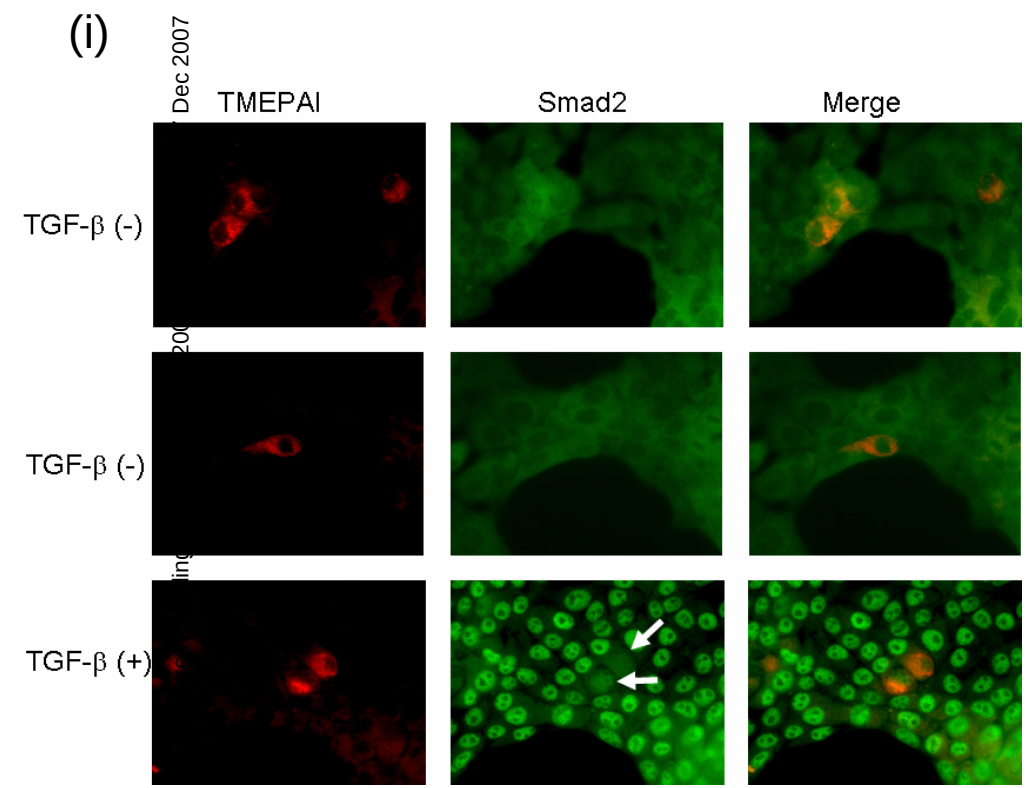

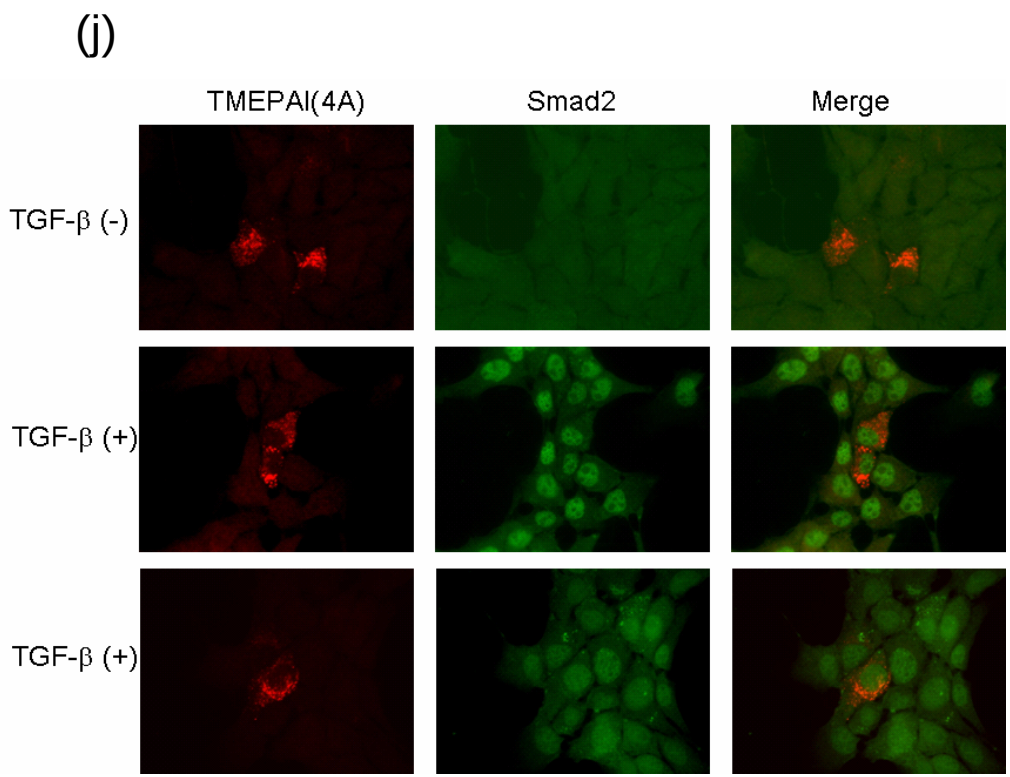

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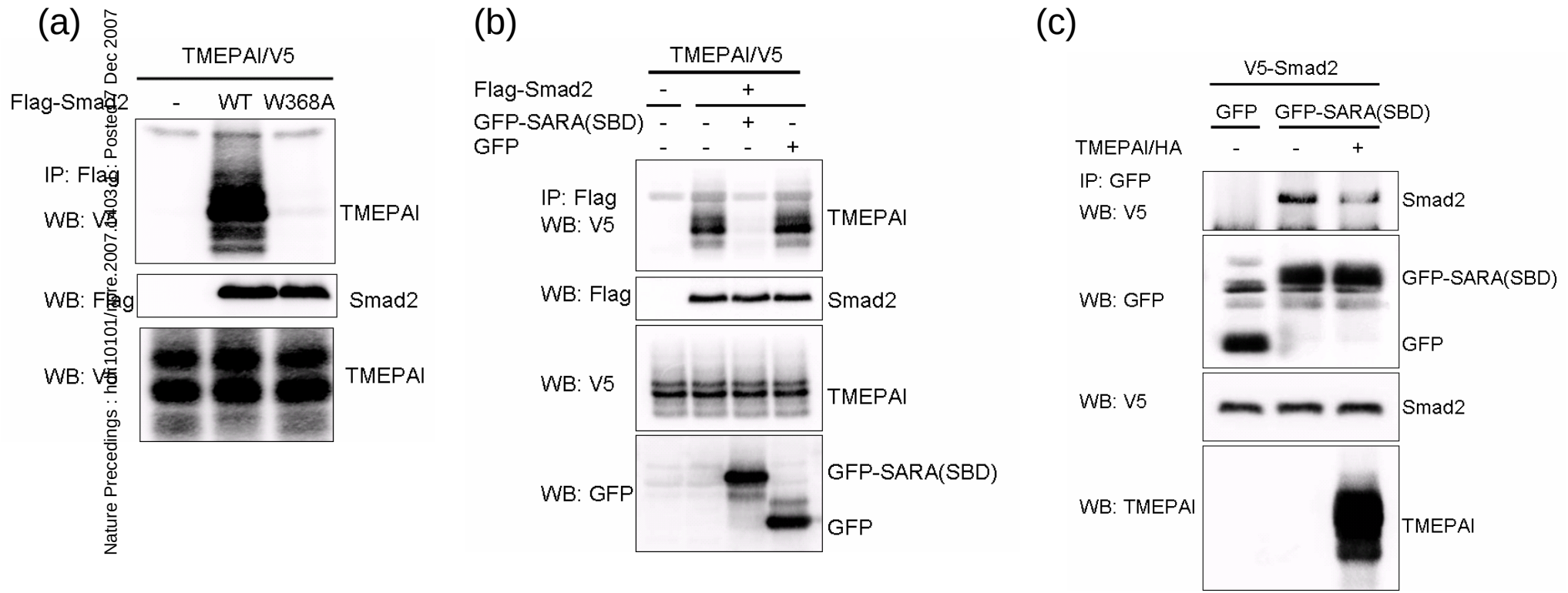

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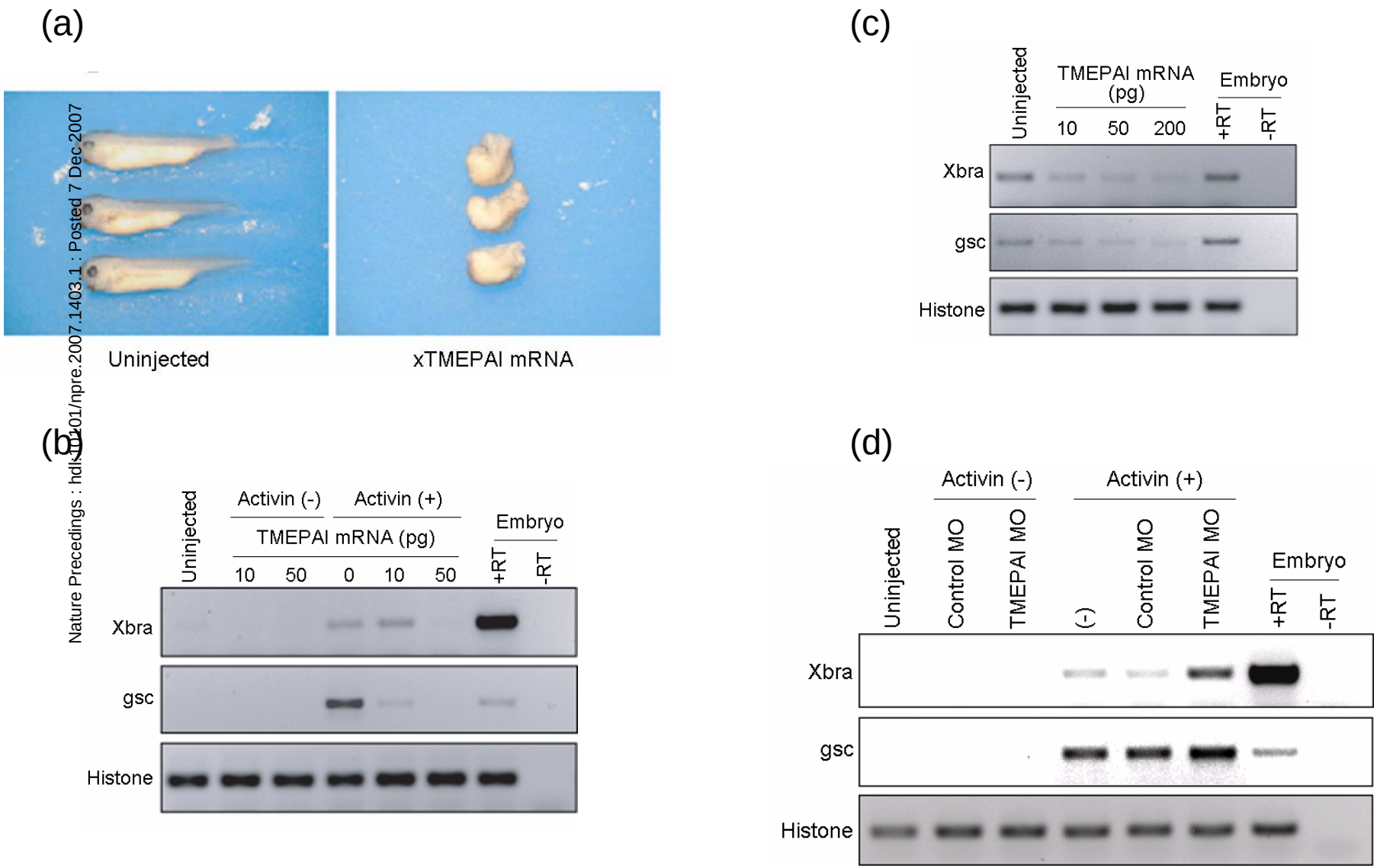

Fig. 7 (Watanabe et al.)