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Identification and Functional Analysis of Zranb2 as a Novel Smad–Binding Protein That Suppresses BMP Signaling

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ABSTRACT

Smads 1/5/8 transduce the major intracellular signaling of bone morphogenetic proteins (BMPs). In the present study, we analyzed Smad1-binding proteins in HEK293T cells using a proteomic technique and identified the protein, zinc-finger, RAN-binding domain-containing protein 2 (ZRANB2). Zranb2 interacted strongly with Smad1, Smad5, and Smad8 and weakly with Smad4. The overexpression of Zranb2 inhibited BMP activities in C2C12 myoblasts *in vitro*, and the injection of Zranb2 mRNA into zebrafish embryos induced weak dorsalization. Deletion analyses of Zranb2 indicated that the serine/arginine-rich (SR) domain and the glutamine-rich domain were required for the inhibition of BMP activity and the interaction with Smad1, respectively. Zranb2 was found to be localized in the nucleus; however, the SR domain-deleted mutant localized to the cytoplasm. The knockdown of endogenous Zranb2 in C2C12 cells enhanced BMP activity. Zranb2 suppressed Smad transcriptional activity without affecting Smad phosphorylation, nuclear localization, or DNA binding. Taken together, these findings suggested that Zranb2 is a novel BMP suppressor that forms a complex with Smads in the nucleus. J. Cell. Biochem. 113: 808–814, 2012. © 2011 Wiley Periodicals, Inc.

KEY WORDS: BMP; SMAD; ZRANB2; PROTEIN INTERACTION; SIGNAL TRANSDUCTION

Bone morphogenetic proteins (BMPs) are multifunctional growth factors that are members of the transforming growth factor-β family. BMPs exhibit a unique activity in bone matrix that is characterized by ectopic bone formation in muscle tissues *in vivo* [Urist, 1965]. BMPs inhibit the myogenic differentiation of myoblasts and cause an osteogenic differentiation into osteoblastic lineage cells *in vitro* [Katagiri et al., 1994]. BMPs physiologically regulate bone formation and the development and regeneration of various tissues in vertebrates and lower animals [Katagiri et al., 2008]. In zebrafish and Xenopus embryos, BMP signaling plays an important role in the determination of a dorsal–ventral axis during development [Suzuki et al., 1994; Nakamura et al., 2007].

BMP signaling is transduced using two different transmembrane serine/threonine kinase receptors, which are termed type I and type II receptors [Miyazono et al., 2005; Wan and Cao, 2005]. The BMP-bound type II receptor phosphorylates the type I receptor. The activated BMP type I receptor kinase subsequently phosphorylates a serine–valine–serine (SVS) motif at the C-termini of Smad1, Smad5, and Smad8, which are the BMP receptor-regulated Smads (R-Smads) [Katagiri, 2010]. The phosphorylated R-Smads form heteromeric complexes with Smad4, which is a common Smad (Co-Smad), and translocate into the nucleus to regulate the transcription of direct target genes, such as *Id1* [Katagiri et al., 2002]. A GC-rich element in the 5′ enhancer region of the *Id1* gene has been identified as the BMP-responsive element (BRE), which is recognized by a complex of BMP-regulated Smads and Smad4 in response to activation of the BMP type I receptor. The overexpression of a constitutively active Smad1, in which the SVS motif has been substituted with a DVD

sequence, activates the expression of the *Id1* reporter gene and the osteoblastic differentiation of myoblasts in the absence of BMPs [Nojima et al., 2010]. Moreover, a chemical inhibitor of BMP type I receptor-mediated R-Smad phosphorylation suppressed BMP receptor-mediated osteoblastic differentiation and heterotopic bone formation [Yu et al., 2008a, b]. These findings suggest that Smads play a critical role in BMP activities both *in vitro* and *in vivo*.

The activity of Smad proteins is regulated via interactions with various factors, including modifying enzymes, co-activators, and co-repressors [Miyazono et al., 2006]. The Smads are degraded by binding to the ubiquitin–proteasome system *via* interactions with E3 ubiquitin ligases, such as Smad ubiquitin regulatory factors 1 and 2 (Smurf1 and Smurf2) [Zhu et al., 1999]. In addition, two distinct phosphatases, small C-terminal domain phosphatases (SCPs) and protein phosphatase magnesium-dependent 1A (PPM1A), have been identified as enzymes that stimulate the dephosphorylation of Smads [Knockaert et al., 2006; Lin et al., 2006]. In the nucleus, Smads interact with various transcriptional co-activators, including p300, CBP, and PCAF [Feng et al., 1998; Itoh et al., 2000], and co-repressors, including Ski, SnoN, and YY1 [Akiyoshi et al., 1999; Wu et al., 2002; Kurisaki et al., 2003]. Thus, the identification and characterization of novel Smad-binding proteins are important to understand the mechanisms of the Smad signaling pathway. In the present study, we report the identification and characterization of a protein, zinc-finger, RAN-binding domain containing protein 2 (Zranb2), as a factor that binds BMP-regulated R-Smads. Zranb2, also known as Zis and Znf265, was identified previously in renal juxtaglomerular cells [Karginova et al., 1997]. Zranb2 contains two RanBP2-type zinc-finger domains, a glutamic acid-rich (Glu) region and a C-terminal Ser/Arg-rich (SR) domain [Mangs and Morris, 2008]. Zranb2 is involved in the alternative splicing of RNA through the zinc-finger domains in a reconstituted assay *in vitro* [Adams et al., 2001]. The current study is the first report to demonstrate that Zranb2 acts as a BMP inhibitor by suppressing Smad transcriptional activity without affecting Smad phosphorylation or DNA-binding capacity.

RESULTS

ZRANB2 INTERACTS WITH BMP-REGULATED SMADS

To identify novel proteins that regulate BMP signaling *via* the Smads, we analyzed Smad1-binding proteins using a proteomic technique. FLAG-Smad1 was overexpressed in HEK293T cells, and the Smad1-binding proteins were enriched from whole-cell extracts using immunoprecipitation with an anti-FLAG antibody. ZRANB2 was identified as a Smad1-binding protein (Fig. 1A). Myc-tagged Zranb2 interacted with FLAG-Smad1 and weakly interacted with FLAG-Smad4 in HEK293T cells (Fig. 1B), and this binding capacity of Zranb2 to Smad1 was not affected by BMP-4 stimulation (Fig. 1C). In addition, Zranb2 interacted with two other BMP-regulated R-Smads, Smad5 and Smad8 (Fig. 1D).

ZRANB2 INHIBITS BMP ACTIVITIES VIA SMADS

We examined the effect of Zranb2 on BMP activities in C2C12 myoblasts, a well-characterized model system for studying the biological activity of BMPs [Katagiri et al., 1994]. The ALP activity
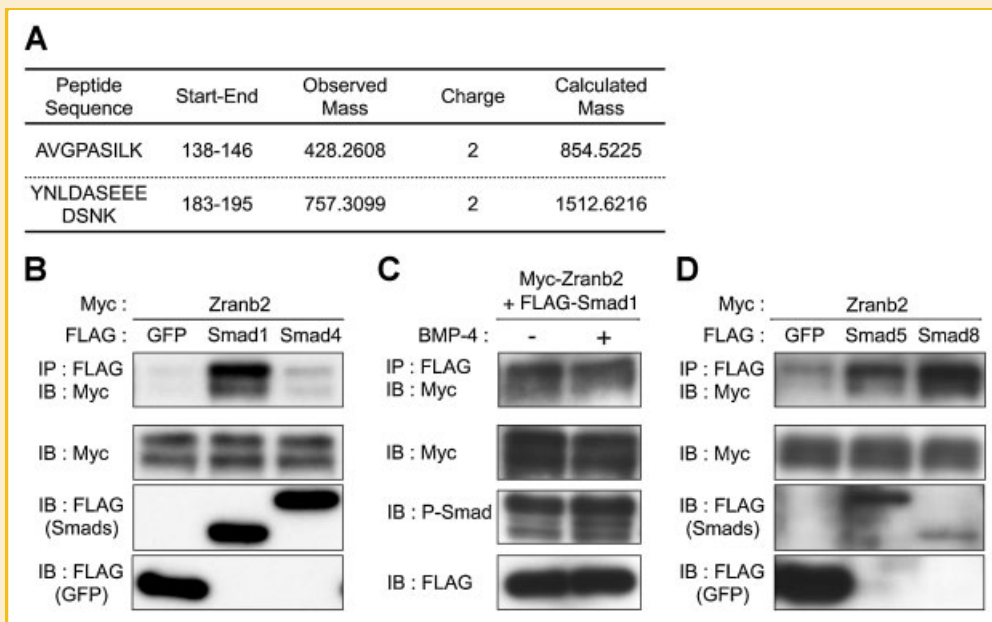

Fig. 1. Zranb2 interacts with Smads. A: Identification of ZRANB2 by LC–MS/MS analysis. The amino acid sequence assigned to each peptide and its position in the ZRANB2 sequence. Also indicates observed mass and charge of the peptide ion together with calculated mass. B, D: HEK293T cells were co-transfected with Myc–tagged Zranb2 and Flag-tagged Smad1 or Smad4 (B), Flag-tagged Smad5 or Smad8 (D) constructs. Whole cell lysates were immunoprecipitated (IP) with an α–FLAG antibody and immunoblotted with an α–Myc antibody. C: HEK293T cells were transfected with Myc–Zranb2 and Flag–Smad1 and treated for 1 h with or without 100 ng/ml BMP–4. Whole cell lysates were IP with an α–FLAG antibody and immunoblotted with an α–Myc antibody.

and *Osterix* mRNA expression were measured as typical markers of the osteoblastic differentiation that is induced by BMP activity. The overexpression of Zranb2 suppressed the ALP activity and osterix mRNA, which were induced by the transient transfection of constitutively active BMPR-IA, BMPR-IA(Q233D), and Smad1 (Fig. 2A,B). The overexpression of Zranb2 also suppressed Id1WT4F luciferase reporter activity that was driven by the BRE in the *Id1* gene following BMP-4 stimulation (Fig. 2C). We observed that Zranb2 exhibited similar suppression profiles on the BMP activity in both osteoblastic MC3T3-E1 cells and primary osteoblasts (data not shown). The injection of synthetic Zranb2 mRNA into *Danio rerio* (zebrafish) embryos caused approximately 4.4% dorsalized embryos, indicating that Zranb2 has a weak BMP-inhibitory effect in developing zebrafish (Fig. 2D,E). This result was surprising since we likely expect approximately more than 50% phenotypic consequences when target genes are mutated in zebrafish [Nakamura et al., 2007]. These results suggest that Zranb2 acts as a suppressor of BMP activity in mammalian cells and in *Danio rerio* embryos.

THE SR DOMAIN OF ZRANB2 IS ESSENTIAL FOR THE INHIBITION OF BMP SIGNALING

The primary sequence of Zranb2 predicts several structurally distinct domains: Two zinc-fingers (ZFs), a glutamic acid (Glu)-rich domain, and a serine and arginine-rich (SR) domain. To identify the domain of Zranb2 that is crucial for BMP inhibition, we generated a series of deletion mutants of Myc-Zranb2 lacking the ZFs and/or the Glu and/or SR domains (Fig. 3A). Our immunohistochemical analysis using an anti-Myc antibody indicated that the ΔZF1, ΔZF, and SR mutants were localized in the nucleus and suppressed the BMP activity similar to the wild-type Zranb2 (Fig. 3B,C). However, the ΔSR mutant was mainly localized in the cytoplasm

and showed a slight enhancement, rather than an inhibition, of BMP signaling (Fig. 3B,C). We further examined the interactions of these mutants with Smad1 in a co-immunoprecipitation assay. Unexpectedly, the ΔSR mutant bound to Smad1 despite the loss of the BMP inhibition (Fig. 3D). In contrast, the SR mutant did not interact with Smad1 but suppressed BMP activity (Fig. 3D). These results suggest that the Glu and SR domains may be required for Smad interactions and BMP inhibition, respectively (see the Discussion section). In addition, we demonstrated that the MH2 domain of Smad1 was a Zranb2-interacting domain (Fig. 3E).

ZRANB2 SUPPRESSES THE TRANSCRIPTIONAL ACTIVITY OF SMADS WITHOUT INHIBITING THEIR DNA-BINDING ACTIVITY

To clarify the molecular mechanisms of Zranb2, we examined the effect of Zranb2 on the early events that are induced by BMP signaling, which include Smad phosphorylation and nuclear localization. Equivalent levels of phosphorylated Smad1/5/8 that were induced by BMPR-IA(Q233D) were detected in the presence and absence of Zranb2 (Fig. 4A). We demonstrated that phosphorylated Smad1/5/8 were localized in the nuclei in Zranb2-negative and Zranb2-overexpressing cells (Fig. 4B). Moreover, Zranb2 suppressed the BMP-specific luciferase reporter activity that was induced by the constitutively activated Smad1, Smad1(DVD), which activates the transcription of target genes, regardless of the phosphorylation status (Fig. 4C). These results suggest that the BMP receptor-mediated phosphorylation of Smad1/5/8 is not the targeted event of Zranb2-mediated suppression.

We also investigated the role of endogenous Zranb2 on the BMP activity in C2C12 cells. Transfection of two types of siRNAs against mouse Zranb2 but not scramble siRNA reduced the Zranb2 protein levels and enhanced the Id1WT4F luciferase reporter activity
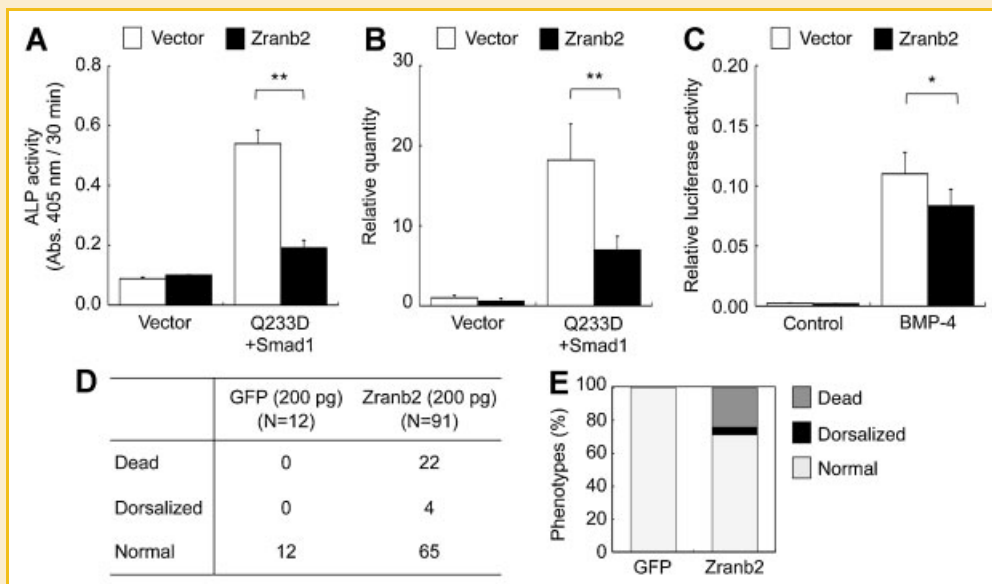

Fig. 2. Zranb2 inhibits BMP activity. A, B: ALP activity (A) or *Osterix* mRNA expression (B) was induced by the overexpression of constitutively active BMPR-IA (Q233D) with Smad1 in the presence or absence of Zranb2 in C2C12 cells. C: IdWT4F-luc activity was induced by BMP-4 (5 ng/ml) in C2C12 cells that were transfected with or without Zranb2. D, E: Zranb2 induces weak dorsalization of the zebrafish embryo; synthetic Zranb2 mRNA (200 pg) was injected into the embryos. Results are presented as the mean ± SD (n = 3), *P < 0.05 and **P < 0.01 compared with control.
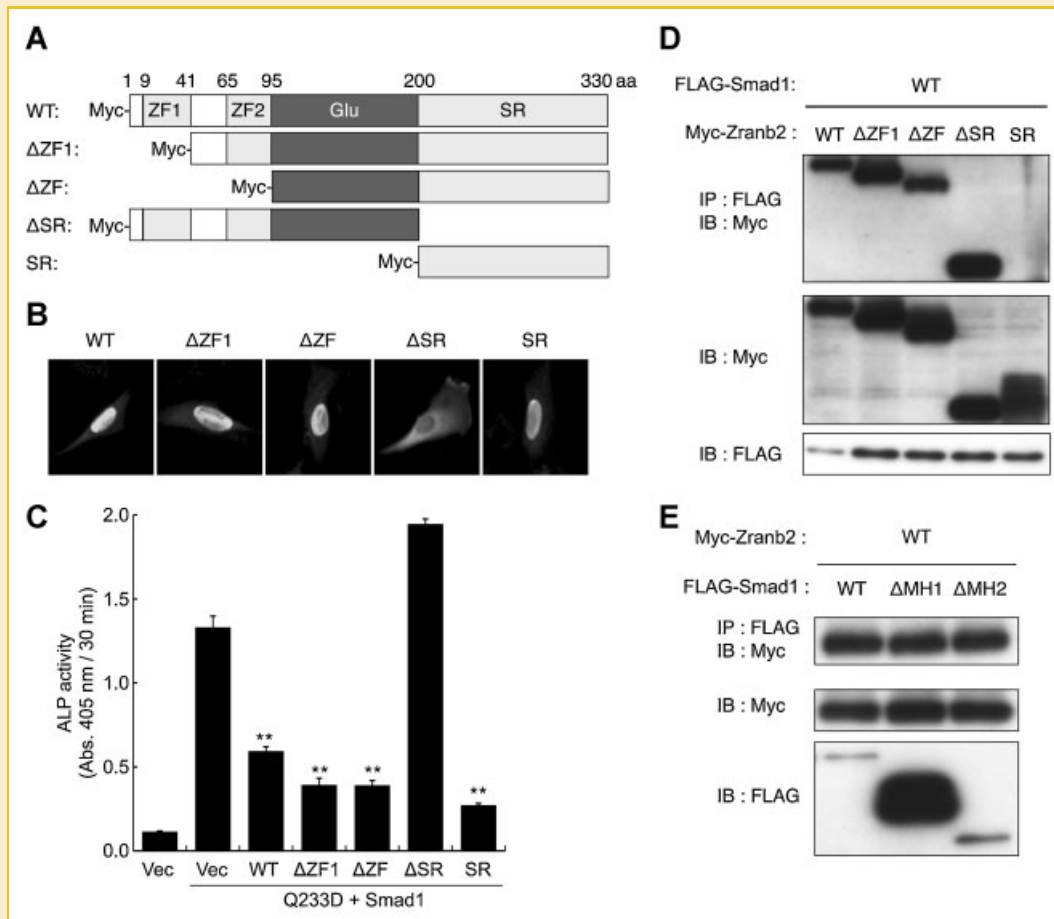
Fig. 3. The SR domain of Zranb2 is important for the inhibition of BMP activity. A: Construction of the deletion mutants of Zranb2. ZF1 and ZF2, zinc finger domains; Glu, a glutamic acid–rich domain; and SR, a serine and arginine–rich domain. B: Cellular localization of the Zranb2 mutants. C2C12 cells were transfected with one of the Zranb2 mutants followed by immunofluorescent staining with an α–Myc antibody. Original magnification, ×40. C: The SR domain suppressed the BMP activity. The ALP activity was induced by the overexpression of BMPR–IA(Q233D) and Smad1 in the presence or absence of Zranb2 in C2C12 cells. D: Analysis of the binding capacities of the Zranb2 mutants to Smad1. HEK293T cells were co-transfected with FLAG–tagged Smad1 and one of the Myc–tagged Zranb2 constructs. Whole-cell lysates were immunoprecipitated with an α–FLAG antibody and immunoblotted with an α–Myc antibody. E: The analysis of the binding capacities of the MH1 and MH2 domains of Smad1 to Zranb2. HEK293T cells were co-transfected with Myc–tagged Zranb2 and Flag–tagged Smad1(ΔMH1) or Smad1(ΔMH2). Whole cell lysates were immunoprecipitated with an α–FLAG antibody and immunoblotted with an α–Myc antibody. Results are presented as the mean ± SD (n = 3), $^*P < 0.05$ and $^{**}P < 0.01$ compared with control.

(Fig. 4D,E). These results suggest that Zranb2 physiologically suppresses BMP activity. However, equivalent amounts of phosphorylated Smad1/5/8 were bound to the BRE in a DNA-precipitation assay in the presence and absence of Zranb2 siRNAs. These results suggest that Zranb2 does not block the DNA-binding capacity of Smads (Fig. 4D). In addition, Zranb2 itself was not found to interact with the BRE (Fig. 4D).

DISCUSSION

In the present study, we identified Zranb2 as a novel inhibitor of BMP activity via interactions with BMP-specific Smads. Zranb2 suppressed the BMP activity in mammalian cells *in vitro* and zebrafish embryos *in vivo*. These results suggest that Zranb2 may act as an inhibitor of BMP activity in vertebrates. This is the first report demonstrating interactions between Zranb2 and BMP signaling.

Zranb2 is an SR domain-containing nuclear protein that is ubiquitously expressed in various tissues and is conserved from nematodes to mammals [Karginova et al., 1997; Mangs and Morris, 2008]. The SR proteins are splicing factors that contain a C-terminal SR domain and an N-terminal RNA recognition ZF motif [Shen and Green, 2006]. Zranb2 recognizes RNA *via* its N-terminal ZF motifs and regulates alternative splicing in a reconstituted model *in vitro* [Loughlin et al., 2009]. However, our deletion study showed that the ZF motifs were not essential for BMP inhibition or Smad1 binding. These results suggest that the alternative splicing of RNA may not be involved in Zranb2-mediated inhibition of BMP activity. In contrast, the SR domain of Zranb2 was essential for the nuclear localization and the inhibition of BMP activity. However, this SR domain did not interact with Smad1. The Glu-rich domain may be involved in interactions with the Smad1 MH2 domain. Although ZRANB1 and ZRANB3 share conserved ZF motifs with ZRNAB2, their amino acid sequences do not show a significant similarity with the Glu-rich
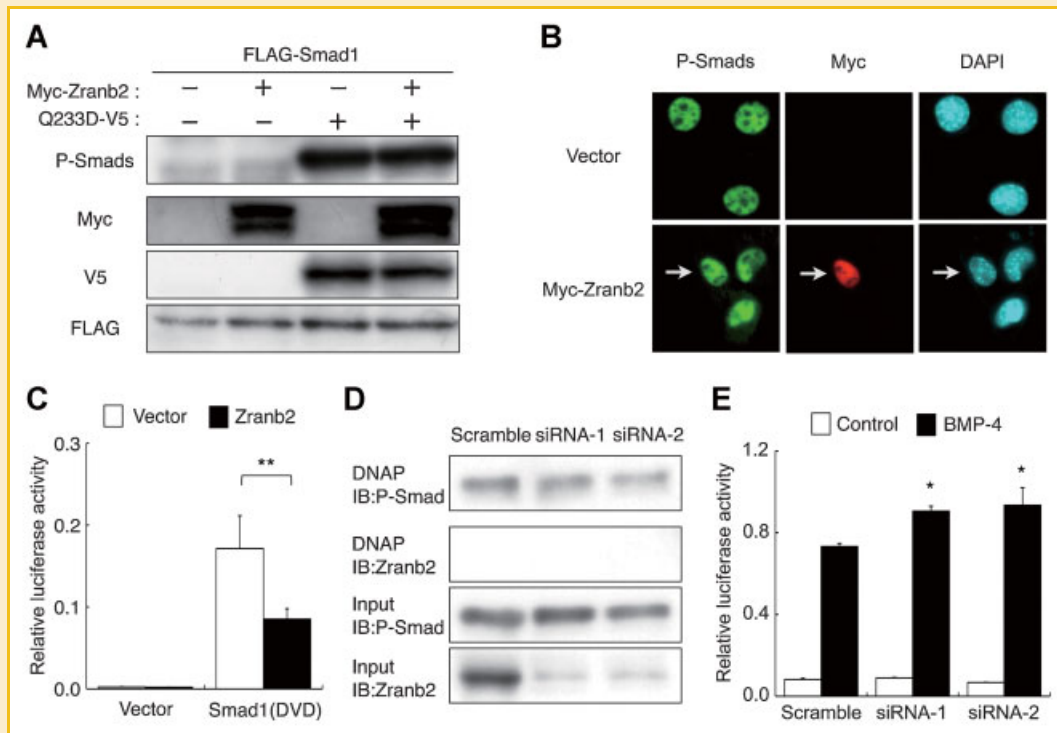
Fig. 4. Zranb2 inhibits the transcriptional activity of Smads without suppressing their DNA-binding capacity. A: Western blot analysis. C2C12 cells were co-transfected with FLAG-tagged Smad1 with or without Myc-tagged Zranb2 and V5-tagged BMPR-IA(Q233D). Whole cell lysates were immunoblotted with α-phospho-Smad1/5/8, α-FLAG, α-Myc, or α-V5 antibodies. B: C2C12 cells were transfected with Myc-tagged Zranb2 or empty vector and treated for 1 h with BMP-4 (100 ng/ml) and stained with α-phospho-Smad1/5/8 or α-Myc antibodies. Original magnification, ×40. C: Zranb2 inhibited the BMP-specific IdWT4F-luc activity that was induced by the constitutively activated Smad1, Smad1DVD. D, E: Effects of Zranb2 siRNA knockdown on the BMP activity and DNA binding of Smads. C2C12 cells were transfected with Zranb2 siRNA or scrambled siRNA. D: Nuclear extracts were affinity precipitated using the BRE and immunoblotted with α-phospho-Smad1/5/8 or α-Zranb2 antibodies. E: BMP-specific luciferase activity was induced by BMP-4. Results are presented as the mean ± SD (n = 3), *$P < 0.05$ and **$P < 0.01$ compared with control.

domain, SR domain, or other regions of ZRANB2 [Evans et al., 2001; Wiemann et al., 2001]. The Glu-rich and SR domains are important for the Zranb2-mediated inhibition of BMP signaling. Therefore, we hypothesized that the inhibition of BMP signaling may be unique to Zranb2 among these ZF motif-containing proteins.

Zranb2 inhibited the BMP-induced osteoblastic differentiation of the C2C12 cells and the BMP-specific luciferase reporter activity that was driven by the BRE in the *Id1* gene. However, Zranb2 did not affect the phosphorylation levels or nuclear localization of the Smads. Moreover, the DNA-binding capacity of Smads in response to BMP stimulation was not changed by the presence or absence of Zranb2, suggesting that another mechanism is involved in Zranb2-mediated inhibition. According to our results, Zranb2 interacts with the Smad1 MH2 domain, which is important for interactions with other co-activators and co-repressors, such as Smad4, p300/CBP, c-Ski, and YY1. A previous study has reported that Zranb2 co-localizes with p300 and YY1 in the nucleus [Adams et al., 2001]. Thus, it may be possible that Zranb2 outcompetes a co-activator that is essential for the transcription of target genes or recruits a co-repressor to the Smad-DNA complex on the BRE. We showed that Zranb2 was not detected on the BRE in a DNAP assay. This result supports the possibility of competition between Zranb2 and co-activators. Further studies are needed to identify and elucidate the detailed

molecular mechanisms, including the critical target molecules of Zranb2 that mediate Zranb2-dependent BMP inhibition.

In conclusion, Zranb2 is a novel BMP suppressor that forms a complex with Smads in the nucleus. Our findings provide new insight into the molecular mechanisms of BMP signaling.

MATERIAL AND METHODS

Immunopurification and mass spectrometry. The FLAG-tagged Smad1 plasmid that is described below was transfected into HEK293T cells. The cytosolic extraction and immunoprecipitation procedures were performed as previously described [Komatsu et al., 2004]. The eluates from the immunoprecipitates were analyzed using nanoscale LC-MS/MS system as described previously [Natsume et al., 2002].

Plasmid constructs. Plasmids encoding the wild-type Smads, constitutively active BMPR-IA(Q233D), constitutively active Smad1(DVD), and IdWT4F-luc have been previously described [Nojima et al., 2010], [Fukuda et al., 2008]. The wild-type and mutated mouse Zranb2 (Accession number NM_017381) derivatives were obtained using standard RT-PCR techniques with PrimeStar HS DNA polymerase (TaKaRa, Shiga, Japan) and cloned into a pcDEF3 expression vector [Goldman et al., 1996]. All of the final constructs were confirmed by sequencing.

Cell culture, transfection, ALP assay, and the luciferase reporter assay. HEK293T human kidney cells, C2C12 mouse myoblasts, C3H10T1/2 mouse fibroblasts, and MC3T3-E1 mouse osteoblasts were maintained as previously described [Kokabu et al., 2011; Ohte et al., 2011]. The cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The ALP activity was measured as a marker of osteoblast differentiation as previously described [Kodaira et al., 2006]. The luciferase reporter assay was performed using IdWT4F-luc and phRL-SV40 (Promega, Madison, WI) with the Dual-Glo Luciferase Assay system (Promega) as previously described [Katagiri et al., 2002].

Immunohistochemistry, western blot, and immunoprecipitation. The following antibodies were used for the immunohistochemistry, immunoprecipitation, and western blot analysis: α-FLAG (clone M2, Sigma, St Louis, MO), polyclonal α-Myc (Medical & Biological Laboratories, Nagoya, Japan), α-V5 (clone V5005, Nacalai Tesque, Kyoto, Japan), polyclonal α-phosphorylated Smad1/5/8 (Cell Signaling Technology, Beverly, MA), polyclonal α-ZNF265(Zranb2) (E-16, Santa Cruz Biotechnology, Heidelberg, Germany), and α-MHC (clone MF-20, Developmental Studies Hybridoma Bank, Iowa City, IA). For the immunohistochemical analysis, the target proteins were visualized using the Alexa488- or Alexa594-conjugated secondary antibodies (Invitrogen). The western blot analysis was performed as previously described [Fukuda et al., 2009]. The target proteins were immunoprecipitated for 3 h at 4°C using M2-agarose beads (Sigma).

Injection of synthetic RNA into zebrafish embryos. The full-length Zranb2 sequence was cloned into the pCS2+ expression vector, and RNA was synthesized from NotI-digested pCS2+ plasmids using the SP6 mMessage mMachine kit (Ambion, Austin, TX). Phenol red (0.1%) was added to the RNA solution as a tracer, and the RNA (200 pg) was injected into 1–2 cell-stage embryos. Following the injection, the embryos were cultured in aquatic system water and imaged as previously described [Nakamura et al., 2007].

DNA affinity precipitation. The DNA affinity precipitation was performed as previously described, with some modifications [Suzuki et al., 1993]. Nuclear extracts from the C2C12 cells were prepared using the ProteoJET cytoplasmic and nuclear protein extraction kit (Fermentas, Glen Burnie, MD). The biotinylated DNA probes were prepared using PCR with biotin-conjugated primers. The biotinylated DNA probe (1 μg) and nuclear extracts (100 μg of protein) were incubated for 30 min on ice in a volume (500 μl) of a solution containing 20 mM HEPES–KOH (pH 7.9), 80 mM KCl, 1 mM MgCl$_2$, 0.2 mM EDTA, 0.5 mM DTT, 5%(v/v) glycerol, protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany), and phosphatase inhibitor (Nacalai Tesque). Dynabeads M-280 streptavidin (Invitrogen) were added and mixed by rotation for 1 h at 4°C. The collected proteins were subjected to western blot analysis as described above.

Reverse transcriptase PCR and real-time PCR analysis. Total RNA was isolated using TRIzol (Invitrogen) and reverse-transcribed into cDNA using Superscript III (Invitrogen). The cDNA was amplified using PCR with specific primers for *Osterix*, *Alp*, *Osteocalcin*, and *Atp5f1* (TaKaRa). *Atp5f1* was used as control.

SYBR green-based real-time PCR was performed in a 96-well plate format SYBR Premix Ex Taq (TaKaRa) and a Thermal Cycler Dice Real-time system TP800 (TaKaRa).

Statistical analysis. An unpaired Student's t-test was used for the comparisons. The data are expressed as the mean ± SD, and the statistical significance is indicated as $^*P < 0.05$ and $^{**}P < 0.01$.

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