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Authors

Yang, Kyung-Min
Kim, Wonjoo
Bae, Eunjin
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DRAK2 Participates in a Negative Feedback Loop to Control TGF-β/Smads Signaling by Binding to Type I TGF-β Receptor

Kyung-Min Yang,[1] Wonjoo Kim,[1] Eunjin Bae,[1] Jungsoo Gim,[3] Brian M. Weist,[5] Yunshin Jung,[1] Ja-Shil Hyun,[1] Jennifer B. Hernandez,[5] Sun-Hee Leem,[6] Taesung Park,[3,4] Joon Jeong,[7] Craig M. Walsh,[5] and Seong-Jin Kim[1,2,*]
[1]CHA Cancer Institute
[2]Department of Biomedical Science
CHA University of Medicine and Science, Seoul 135-081, Korea
[3]Interdisciplinary Program in Bioinformatics
[4]Department of Statistics
Seoul National University, Seoul 151-742, Korea
[5]Department of Molecular Biology and Biochemistry, University of California, Irvine, Irvine, CA 92697-3900, USA
[6]Department of Biology and Biomedical Science, Dong-A University, Busan 604-714, Korea
[7]Department of Surgery, Gangnam Severance Hospital, Yonsei University Medical College, Seoul 135-720, Korea
*Correspondence: kimsj@cha.ac.kr
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SUMMARY

TGF-β1 is a multifunctional cytokine that mediates diverse biological processes. However, the mechanisms by which the intracellular signals of TGF-β1 are terminated are not well understood. Here, we demonstrate that DRAK2 serves as a TGF-β1-inducible antagonist of TGF-β signaling. TGF-β1 stimulation rapidly induces DRAK2 expression and enhances endogenous interaction of the type I TGF-β receptor with DRAK2, thereby blocking R-Smads recruitment. Depletion of DRAK2 expression markedly augmented the intensity and the extent of TGF-β1 responses. Furthermore, a high level of DRAK2 expression was observed in basal-like and HER2-enriched breast tumors and cell lines, and depletion of DRAK2 expression suppressed the tumorigenic ability of breast cancer cells. Thus, these studies define a function for DRAK2 as an intrinsic intracellular antagonist participating in the negative feedback loop to control TGF-β1 responses, and aberrant expression of DRAK2 increases tumorigenic potential, in part, through the inhibition of TGF-β1 tumor suppressor activity.

INTRODUCTION

Transforming growth factor-β1 (TGF-β1) is a multifunctional cytokine that regulates cell proliferation, differentiation, migration, and apoptosis (Massagué et al., 2000; Massagué, 2008). TGF-β1 signals through type I and type II serine/threonine kinase receptors (TβRI and TβRII) and their intracellular mediators known as Smad proteins. TGF-β1 ligand initially binds to TβRII and recruits TβRI, thereby forming heteromeric complexes between TβRI and TβRII. In TβR complexes, TβRII phosphorylates TβRI, leading to the activation of a TβRI kinase domain, which, in turn, phosphorylates the receptor-associated Smads (R-Smads; Smad2 and Smad3) at their C-terminal serine residues. Phosphorylated R-Smads form heteromeric complexes with common mediator Smad (Co-Smad; Smad4) and then translocate to the nucleus where they act as a transcriptional regulator for TGF-β1 target genes (Shi and Massagué, 2003; Feng and Derynck, 2005; Massagué et al., 2005). Beside R-Smads and Co-Smad, the inhibitory Smads (I-Smads; Smad6 and Smad7) play key roles in TGF-β signaling, providing negative regulation through interaction of TβRI (Hayashi et al., 1997).

Dysregulation of negative feedback of TGF-β signaling influences tumor growth. Among the negative regulators, all of which are direct target genes of TGF-β signaling, I-Smads, transmembrane prostate androgen-induced RNA (TMEPAI), Ski-related novel protein N (SnoN), and Smad ubiquitination regulatory factors (Smurfs) play key roles in the negative regulation of TGF-β signaling (Itoh and ten Dijke, 2007; Watanabe et al., 2010). Overexpression of Smad7 has been recently shown to promote inhibition of TGF-β signaling in human pancreatic, colorectal, skin, and breast cancers (Yan et al., 2009). Moreover, it has been reported that TMEPAI inhibits TGF-β signaling by interfering TβRI-mediated R-Smads phosphorylation, thus antagonizing the tumor-suppressive effects of TGF-β1 in breast cancer (Watanabe et al., 2010). Therefore, disruption of TGF-β signaling caused by elevated levels of its negative regulators can lead to the development of TGF-β1-associated human cancers.

DRAK2 (DAP kinase-related apoptosis-inducing protein kinase 2), also referred to as STK17B, is known as a negative regulator for T cell activation (Sanjo et al., 1998; McGargill et al., 2004). It is a serine/threonine kinase belonging to the death-associated protein kinase (DAPK) family and is identified as a proapoptotic protein kinase (Bialik and Kimchi, 2006). However,

among the DAPK family, the role of DRAK2 in the regulation of apoptosis is still controversial. In one transgenic mouse model, DRAK2 induces increased T cell apoptosis and aggravated β cell apoptosis when triggered by apoptosis-inducing stimuli, whereas *drak2*-deficient mice did not show any defects in apoptosis and retroviral expression of DRAK2 did not lead to the increase of apoptosis in NIH 3T3 or T cells (Kuwahara et al., 2006; Mao et al., 2006, 2009; McGargill et al., 2004). Although the physiological functions of DRAK2 have been extensively examined in the thymus, many aspects regarding this kinase, including the significance of its expression, intracellular localization, and binding partners and catalytic substrates remain to be elucidated in other tissues.

Here, we identify a role for DRAK2 as an antagonist of TGF-β signaling, which may act in the negative feedback loop to regulate the intensity or the duration of the TGF-β signal. DRAK2 specifically interacted with TβRI, thereby blocking the phosphorylation of Smad2/3, which is the core process in the canonical TGF-β signaling. DRAK2 was highly expressed in basal-like and HER2-enriched human breast tumors as well as in the basal-like breast cancer cell lines. Furthermore, loss of DRAK2 expression inhibited tumor growth in a xenograft model, suggesting that increased expression of DRAK2 may promote tumorigenesis by constraining the TGF-β1 tumor suppressor activity.

RESULTS

DRAK2 Specifically Interacts with TβRI

We performed mass spectrometry-based proteomic screen of TGF-β receptor-associated proteins in HEK293 cells stably expressing HA-tagged TGF-β receptors to identify novel binding partners of TGF-β receptors (Figure 1A). Sequence analysis showed that one of these peaks identified by mass spectrometry contained DRAK2. To confirm the interaction of TβRI with human DRAK2, 293T cells were cotransfected with Myc-tagged DRAK2 and HA-tagged TβRI and then treated with or without TGF-β1. DRAK2 specifically interacted with TβRI (Figure 1B). Although subcellular localization of DAPK family members is dependent on the cell type, only DRAK2 is known to localize to both the cytosol and the nucleus (McGargill et al., 2004). Next, we examined the subcellular distribution of DRAK2 and TβRI in HeLa cells. Comparison of the subcellular distribution of DRAK2 and TβRI by confocal microscopy revealed extensive overlap (Figure 1C). We also confirmed the interaction or colocalization between DRAK2 and TβRI by in situ proximity ligation assay (PLA), a specific and highly sensitive method for detecting this protein-protein interaction. The signals from the proximally localized proteins are amplified as red dots in the in situ PLA. Confocal imaging showed that DRAK2 interacted with TβRI on the cell membrane as well as in the cytosol (Figure 1D). To further elaborate the physiological significance of the interaction between DRAK2 and TβRI, we examined the endogenous interaction of DRAK2 with TβRI. As expected, endogenous DRAK2 interacted with endogenous TβRI in anti-TβRI immunocomplexes (Figure 1E). As ectopic expression of TβRI could form a complex with the endogenous TβRII even in the absence of the ligand, we examined whether the interaction between DRAK2 and TβRI requires TβRII using SNU638 gastric cancer cell line, which is known to be TβRII deficient (Chang et al., 1997). When TβRI was immunoprecipitated by anti-TβRI antibody, endogenous DRAK2 directly bound to TβRI without the help of TβRII (Figure 1F). Interestingly, the interaction between DRAK2 and TβRI was increased by TGF-β1 treatment (Figure 1B). Based on the fact that TβRI can be activated by TβRII only through the formation of heteromeric complexes between TβRI and TβRII, we investigated whether kinase function of TβRI is required for its interaction with DRAK2. We used a series of TβRI mutants (constitutively active mutant; CA, kinase dead mutant; KD, and Smad-binding region mutant; mL45) to examine DRAK2-binding capability. When coexpressed with a series of TβRI mutants, DRAK2 bound to wild-type as well as to each type of the TβRI mutant examined (Figure 1G). We also observed that the interaction between DRAK2 and TβRI was increased depending on the degree of kinase activity of TβRI. Interestingly, mL45 mutant of TβRI, which has lost the ability to interact with Smads due to the mutation in the L45 loop of the kinase domain (Yu et al., 2002), was capable of binding to DRAK2, suggesting that DRAK2 might bind to the regions other than Smads-binding regions of TβRI. To determine the minimal binding domain of TβRI, we designed a glutathione S-transferase (GST) pull-down assay with GST-fused TβRI cytoplasmic domain deletion mutants. DRAK2 was only able to bind to a TβRI kinase domain, but not to a GS domain alone (Figure 1H). To further define the domain in TβRI responsible for DRAK2 binding, we generated a series of deletion mutants of a TβRI kinase domain fused to GST and performed GST precipitation assays. DRAK2 was associated with TβRI kinase domain deletion mutants containing amino acids 216–220, whereas a TβRI kinase domain deletion mutant lacking residues 216–220 failed to interact with DRAK2 (Figure 1I), suggesting that residues 216–220 in TβRI are critical for interaction with DRAK2. Next, we generated single or double amino-acid-substitution mutants of TβRI cytoplasmic domain (216–220) fused to GST (Figure 1J and S1). Single amino-acid-substitution mutants in residues 216–224 did not affect binding of TβRI (149–450) to DRAK2 (Figure S1). However, substitution of the Val-Trp at positions 219–220 with Ala-Ala reduced interactions of TβRI (149–450) to DRAK2 (Figure 1J).

DRAK2 Suppresses TGF-β Signaling

To investigate whether the interaction of DRAK2 with TβRI plays a role in TGF-β signaling, we tested the effect of DRAK2 expression on TGF-β signaling using TGF-β1-responsive reporters (SBE-Luc and 3TP-Luc) in various TGF-β1-responsive cell lines, including Hep3B, HepG2 (hepatocellular carcinoma), and HeLa (cervical cancer) cells. Overexpression of DRAK2 significantly decreased the TGF-β1-induced transcriptional activity of either SBE-Luc or 3TP-Luc (Figures 2A, S2A, and S2B). To further investigate the functional significance of DRAK2 in TGF-β signaling, we performed gain-of-function experiments by generating lentivirus-transduced stable cell lines expressing DRAK2 protein. Overexpression of DRAK2 significantly inhibited the TGF-β1-induced Smad2 and Smad3 phosphorylation (Figures 2B, 2C, S2C, and S2D) and TGF-β1-induced translocation of Smad2 and Smad3

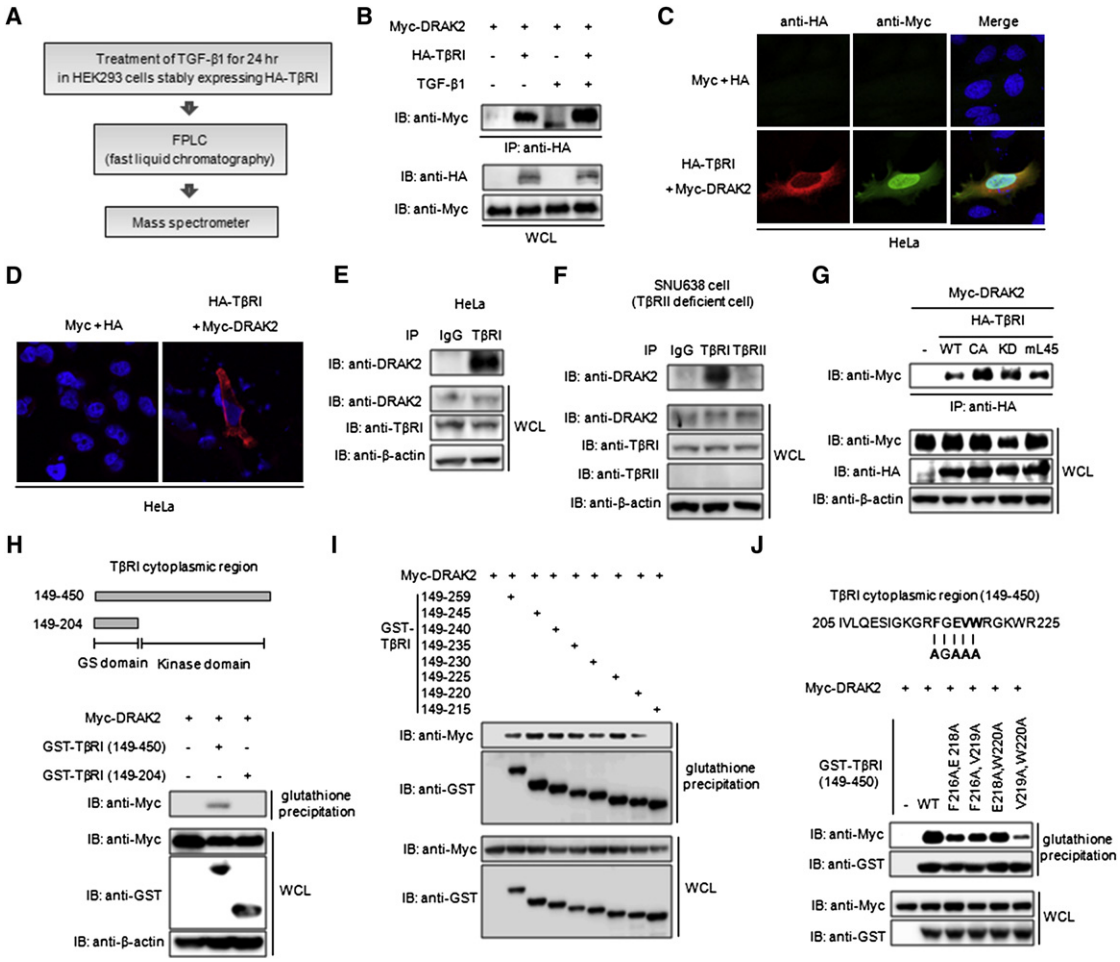

Figure 1. DRAK2 Specifically Interacts with TβRI

(A) The protocol of a TβRI-binding-partner screen using purification methods.

(B) 293T cells were cotransfected with Myc-tagged DRAK2 and HA-tagged TβRI with or without TGF-β1 for 2 hr. Cell lysates were immunoprecipitated with anti-HA antibody and analyzed by immunoblots using anti-HA and anti-Myc antibodies.

(C) Myc-tagged DRAK2 and HA-tagged TβRI were cotransfected in HeLa cells. After transfection, cells were fixed for immunofluorescence. Ectopic expression of DRAK2 and TβRI was visualized using confocal microscopy (green and red, respectively).

(D) Interactions between Myc-tagged DRAK2 and HA-tagged TβRI expressed in HeLa cells were visualized by in situ proximity ligation assay (in situ PLA). In situ PLA signals were visualized in red.

(E) Cell lysates from HeLa cells were immunoprecipitated with antinormal IgG or anti-TβRI antibodies and subjected to immunoblots with anti-DRAK2 antibody.

(F) SNU638 gastric cancer cells were subjected to whole-cell lysis followed by SDS-PAGE, and cell lysates were immunoprecipitated with antinormal IgG, anti-TβRI, or anti-TβRII antibodies and then detected by immunoblots.

(G) 293T cells were cotransfected with Myc-tagged DRAK2 and HA-tagged TβRI wild-type (WT), kinase active (CA), kinase dead form (KD), or Smad binding defective (mL45) and then immunocomplexes of DRAK2 and TβRI mutant constructs were captured using anti-Myc antibody.

(H) Immunoblot analysis of 293T cells cotransfected with plasmids encoding GST-fused TβRI containing its full cytoplasmic region (149–450) or the GS domain (149–204), and Myc-tagged DRAK2. Cells were collected and lysates were mixed with glutathione agarose beads; bound proteins were identified by immunoblot analysis with anti-Myc antibody.

(I) GST pull-down assay of Myc-tagged DRAK2 bound to GST-fused deletion mutants of TβRI.

(J) GST pull-down assay of Myc-tagged DRAK2 bound to GST-fused double amino-acid-substitution mutant TβRI.

See also Figure S1.

to the nucleus in confocal microscopy and subcellular fractionation (Figures 2D and 2E). To verify a function of DRAK2 as a negative regulator of TGF-β signaling in vivo, we monitored the expression of TGF-β1 target genes such as $p15^{Ink4b}$, $p21^{Waf1/Cip1}$, *PAI1*, and *IL11* by RT-PCR in HaCaT cells that stably overexpress DRAK2. These results demonstrated that DRAK2-overexpressing cells responded with diminished sensitivity to TGF-β1 in terms of induction of TGF-β1 target genes (Figure 2F).

Next, we investigated the significance of DRAK2-binding motif in TβRI in TGF-β signaling. We generated the TβRI expression construct lacking DRAK2-binding site. Then, HeLa cells were

cotransfected with HA-tagged TβRI WT or TβRI V219A/W220A mutant and Myc-tagged DRAK2. DRAK2 inhibited TGF-β1-induced Smad3 phosphorylation and transcriptional activity in TβRI WT-transfected cells. However, DRAK2 failed to block TGF-β1-induced Smad3 phosphorylation in the presence of TβRI mutant lacking DRAK2-binding site. The inhibitory activity of TGF-β1-induced reporter activity by DRAK2 was also markedly reduced in the presence of TβRI mutant lacking DRAK2-binding site (Figures 2G and 2H). This result indicates that DRAK2 inhibits TGF-β signaling through interaction with residues 219–220 in TβRI. Interestingly, overexpression of TβRI V219A/W220A mutant slightly enhanced TGF-β1-induced reporter activity, suggesting that the inhibitory activity of TGF-β signaling by endogenous DRAK2 may be relieved by the expression of the TβRI mutant lacking DRAK2-binding site. The TβRI region required for the interaction with DRAK2 appears to be conserved among other type I receptors of TGF-β family (Figure S3A). To confirm whether DRAK2 interacts with other type I receptors of TGF-β family, we performed the immunoprecipitation assay. As expected, DRAK2 also interacted with all type I receptors of TGF-β family (Figure S3B). To examine the possibility whether DRAK2 inhibits activin or bone morphogenetic protein (BMP) signaling, we investigated the effect of DRAK2 expression on activin or BMP signaling using activin- or BMP-response reporters (ARE- or BMP-Luc). Overexpression of DRAK2 decreased the TGF-β1-induced transcriptional activity of ARE-Luc and BMP2-induced transcriptional activity of BMP-Luc (Figures S3C and S3D). Therefore, it is likely that DRAK2 also inhibits signaling of activin or BMP.

We next sought to elucidate the underlying mechanism by which DRAK2 suppresses TGF-β/Smads signaling via its association with TβRI. We first investigated whether DRAK2 blocks complex formation between TβRI and TβRII. Ectopic expression of DRAK2 led to its binding with TβRI, but this binding did not inhibit the interaction between TβRI and TβRII (Figure S4A). We also tested whether DRAK2 suppresses the phosphorylation of Smad2 and Smad3 through direct binding to either of them. However, DRAK2 did not interact with either Smad2 or Smad3 (Figure S4B). Therefore, we examined whether DRAK2 interrupts Smad2/3 and TβRI complex formation, which is critical in boosting cellular TGF-β1 responses. We found that ectopic expression of DRAK2 diminished TGF-β1-induced interaction of TβRI and Smad3. Furthermore, the level of Smad3 bound to active TβRI (HA-Alk5 ca) was inversely related to the level of DRAK2 expression (Figure 2I). Taken together, these data suggest that interaction between DRAK2 and TβRI results in an interruption of complex formation between TβRI and Smad2/3, thereby contributing to the downregulation of Smad2/3-dependent cellular responses.

Kinas Activity of DRAK2 Is Required for Inhibiting TGF-β Signaling

As DRAK2 is a serine/threonine kinase and is able to autophosphorylate, we tested whether the kinase activity of DRAK2 is required for the suppression of TGF-β signaling. We generated a kinase-inactive mutant of DRAK2 by replacing lysine 62, ATP-binding site in the kinase domain, with alanine (Friedrich et al., 2007). To confirm whether DRAK2 can autophosphorylate in our hands, we examined DRAK2 kinase activity in 293T cells transfected with Myc-tagged DRAK2 wild-type (WT) or Myc-tagged DRAK2 K62A form. We incubated the lysates in a kinase assay buffer containing ^{32}P-ATP; DRAK2 WT autophosphorylated, whereas DRAK2 K62A failed to autophosphorylate itself (Figure 3A). To determine whether autophosphorylation of DRAK2 is required for the interaction of DRAK2 with TβRI, we performed an immunoprecipitation assay in 293T cells cotransfected with either Myc-tagged DRAK2 WT or Myc-tagged DRAK2 K62A with HA-tagged TβRI. DRAK2 WT interacted with TβRI, whereas kinase inactivation of DRAK2 decreased its interaction with TβRI (Figure 3B). The ability of DRAK2 to suppress TGF-β1-responsive reporter activity and Smad2/3 phosphorylation induced by TGF-β1 was also markedly attenuated by a functional loss of DRAK2 as well (Figures 3C and 3D). These results suggest that DRAK2 autophosphorylation may alter the protein conformation to allow its interaction with TβRI. DRAK2 homodimerization was then investigated as a mechanism required for the autophosphorylation of DRAK2. Myc-tagged DRAK2 WT was observed only in cells cotransfected with GST-fused and Myc-tagged DRAK2 WT but not in cells cotransfected with GST- or Myc-tagged DRAK2 K62A (Figure 3E). These results demonstrate that only wild-type DRAK2 monomers are capable of dimerization in vivo, suggesting that homodimerization requires the autocatalytic activity of DRAK2. Since DRAK2 is a serine/threonine kinase, to test whether TβRI is a DRAK2 substrate, human GST-tagged recombinant TβRI, TβRII, and DRAK2 proteins were generated for in vitro kinase assay. We confirmed that recombinant DRAK2 protein failed to *trans*-phosphorylate TβRI or TβRII proteins (data not shown).

DRAK2 May Be Oncogenic in Human Breast Cancer

Based on the Oncomine (http://www.oncomine.org) and BioGPS database (http://www.biogps.org), which revealed relatively higher expression of DRAK2 in Hs578T breast cancer cell lines than other cancer cells, we investigated the expression level of DRAK2 in eight human breast cancer cell lines by quantitative RT-PCR. DRAK2 was highly expressed in the basal-like breast cancer cell lines (Hs578T, MDA-MB-231, and MDA-MB-435) compared to the luminal-like breast cancer cell lines (SK-BR-3, ZR75B, BT-474, MCF-7, and T47D) (Figure 4A). We also analyzed the expression of DRAK2 mRNA in different breast tumor subtypes (normal-like, luminal A, luminal B, basal-like, and HER2-enriched) using published microarray data sets (Wang et al., 2005). Interestingly, a significantly embellished level of *DRAK2* expression was observed in basal-like and HER2-enriched tumors compared to normal and luminal types (Figures 4B and 4C). We next tested the expression level of *DRAK2* in normal and primary human triple-negative breast tumor tissues. RT-PCR analyses demonstrated increased expression of DRAK2 mRNA in triple-negative breast tumor tissues compared to normal tissues (Figure 4D). We also performed immunohistochemistry using anti-DRAK2-specific antibody to investigate the expression of DRAK2 in tumor and stromal compartments of breast carcinomas. Expression of DRAK2 is mainly enriched in

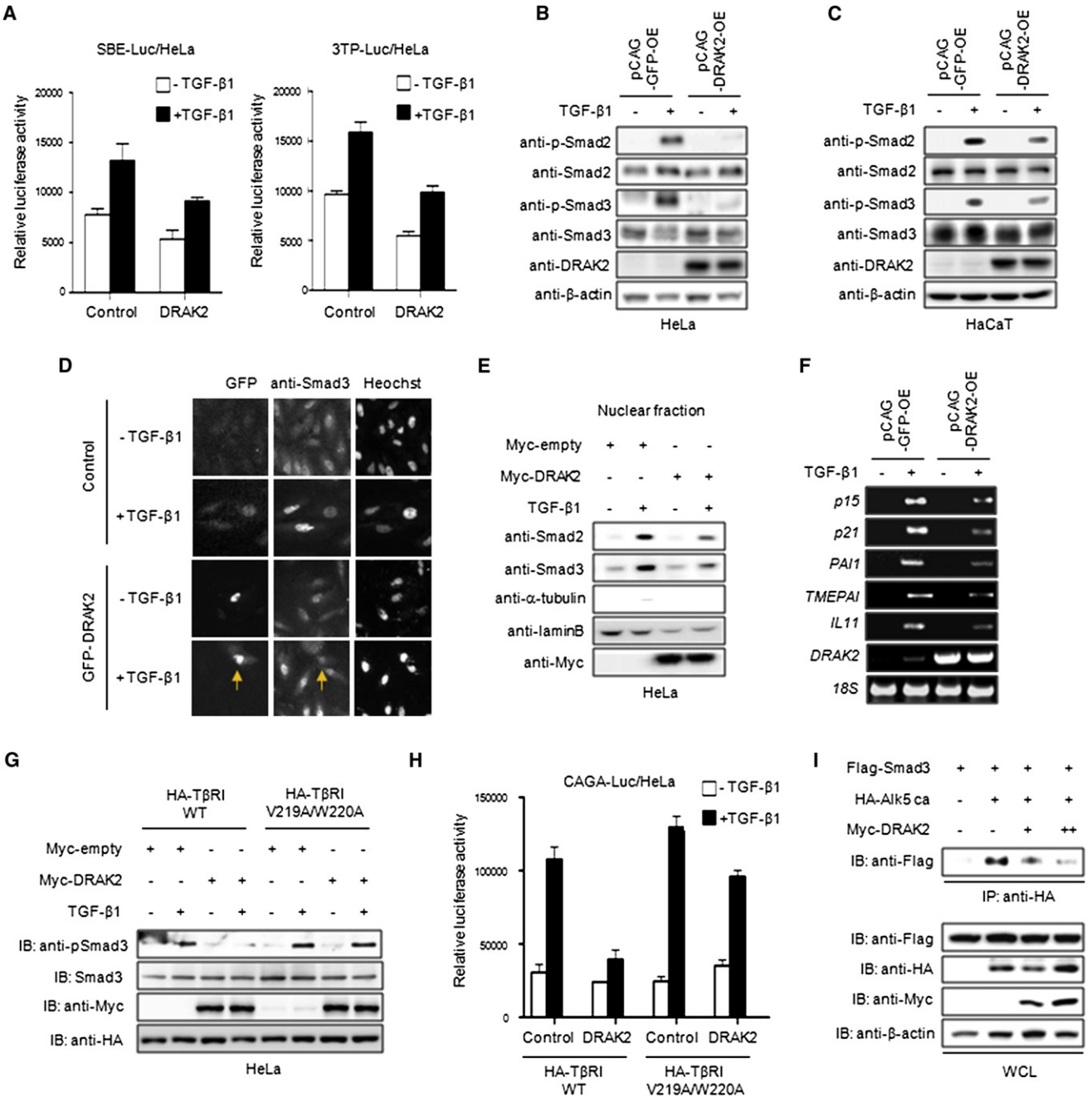

Figure 2. Overexpression of DRAK2 Suppresses the TGF-β Signaling Pathway

(A) Luciferase assay from lysates of HeLa cells cotransfected with TGF-β1-responsive reporters SBE- (left) or 3TP- (right), and Myc-tagged DRAK2, after incubation for 16 hr with or without TGF-β1 treatment. Error bars indicate standard deviation of the mean (SDM) of three independent experiments.

(B and C) HeLa and HaCaT cells infected with GFP-expressing lentivirus (pCAG-GFP) or DRAK2-expressing lentivirus (pCAG-DRAK2) were treated with TGF-β1 for 1 hr. Subsequently, total cell lysates were prepared for immunoblot analysis.

(D) GFP-tagged DRAK2 was transfected in HeLa cells. After transfection, cells were treated with or without TGF-β1 for 2 hr and then fixed for immunofluorescence. Nuclear translocation of Smad3 was detected using anti-Smad3 antibody.

(E) HeLa cells transiently transfected with Myc-tagged DRAK2 were treated with or without TGF-β1 for 2 hr, and then nuclear proteins were subjected to immunoblots.

(F) HaCaT cells stably expressing GFP or DRAK2 protein were treated with TGF-β1 for 8 hr, and then total RNA was isolated. mRNA levels of TGF-β1 target genes were detected by RT-PCR.

(G) HeLa cells were transfected with HA-tagged TβRI WT or lacking DRAK2-binding site and Myc-tagged DRAK2. After transfection, cells were treated with TGF-β1 for 1 hr and then subjected to immunoblots.

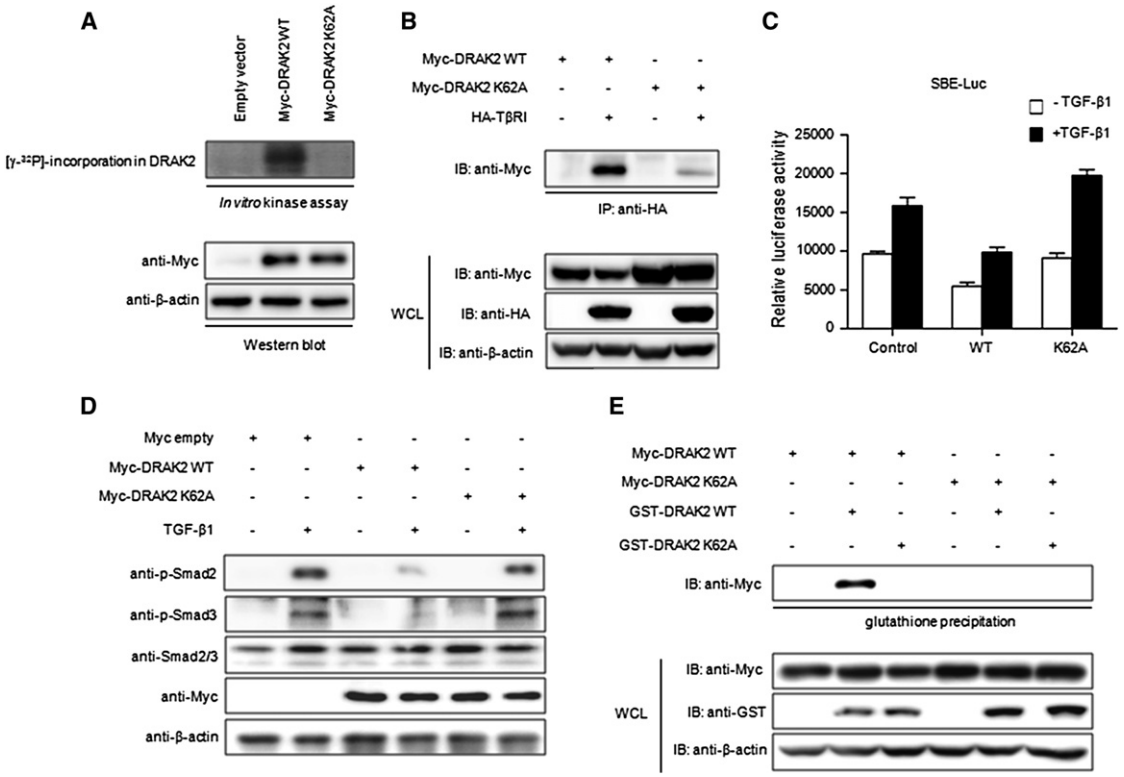

Figure 3. Functional Loss of DRAK2 Enhances TGF-β Signaling

(A) DRAK2 kinase activity was measured in 293T cells transfected with empty vector, Myc-tagged DRAK2, or Myc-tagged kinase inactive DRAK2 (K62A). Cell lysates were incubated in a kinase assay buffer containing ^{32}P-ATP followed by an in vitro kinase assay.

(B) 293T cells were cotransfected with Myc-tagged DRAK2, or Myc-tagged DRAK2 K62A, and HA-tagged TβRI. Cell lysates were immunoprecipitated with anti-HA antibody and then immunoblotted with indicated antibodies.

(C) Luciferase assays of lysates from HeLa cells were performed using cotransfection of the TGF-β1-responsive reporter CAGA-Luc and control vector, Myc-tagged DRAK2, or Myc-tagged DRAK2 K62A, after incubation for 16 hr with or without TGF-β1 treatment. Error bars indicate SDM of three independent experiments.

(D) HeLa cells were cotransfected with control vector, Myc-tagged DRAK2, or Myc-tagged DRAK2 K62A in the presence or the absence of TGF-β1 for 1 hr. Total cell lysates were prepared for immunoblot analysis.

(E) Lysates of 293T cells cotransfected with Myc-tagged DRAK2 WT, GST-fused DRAK2 WT, Myc-tagged DRAK2 K62A, and/or GST-fused DRAK2 K62A were subjected to GST pull-down assays and then detected by immunoblots.

the tumor compartment of breast carcinomas, possibly representing two populations of cells (Figure 4E). Notably, strong nuclear localization of DRAK2 was seen in the epithelial tumor cell compartment, suggesting an unknown role of DRAK2 in the nucleus. Next, based on clinical annotations available from Gene Expression Omnibus (GEO), we analyzed the relapse-free survival of breast cancer patient cohorts using public microarray-based breast cancer data sets (Bos et al., 2009). Patients with high *DRAK2* expression were associated with a significantly shorter relapse-free survival time ($p = 0.0063$) (Figure S5). Taken together, these studies revealed that tumor-associated DRAK2 was predominantly produced by malignant breast cancer cells.

DRAK2 Knockdown Enhances TGF-β Signaling in Breast Cancer Cells

Since Hs578T and MDA-MB-231 breast cancer cells exhibited the highest expression of DRAK2 among the breast cancer cell lines tested, we hypothesized that DRAK2 may inhibit the TGF-β signal through interaction with TβRI in vivo in these breast cancer cell lines. To examine an interaction of DRAK2 with TβRI, immunoprecipitation experiments were performed. We

(H) Luciferase assays of lysates from HeLa cells were performed using cotransfection of the TGF-β1-responsive reporter CAGA-Luc, Myc-tagged DRAK2, and HA-tagged TβRI WT or lacking DRAK2-binding site, after incubation for 16 hr with or without TGF-β1 treatment. Error bars indicate SDM of three independent experiments.

(I) 293T cells were cotransfected with indicated plasmids and harvested for immunoprecipitation. Bound proteins were identified with anti-HA and anti-Flag antibodies.

See also Figures S2, S3, and S4.

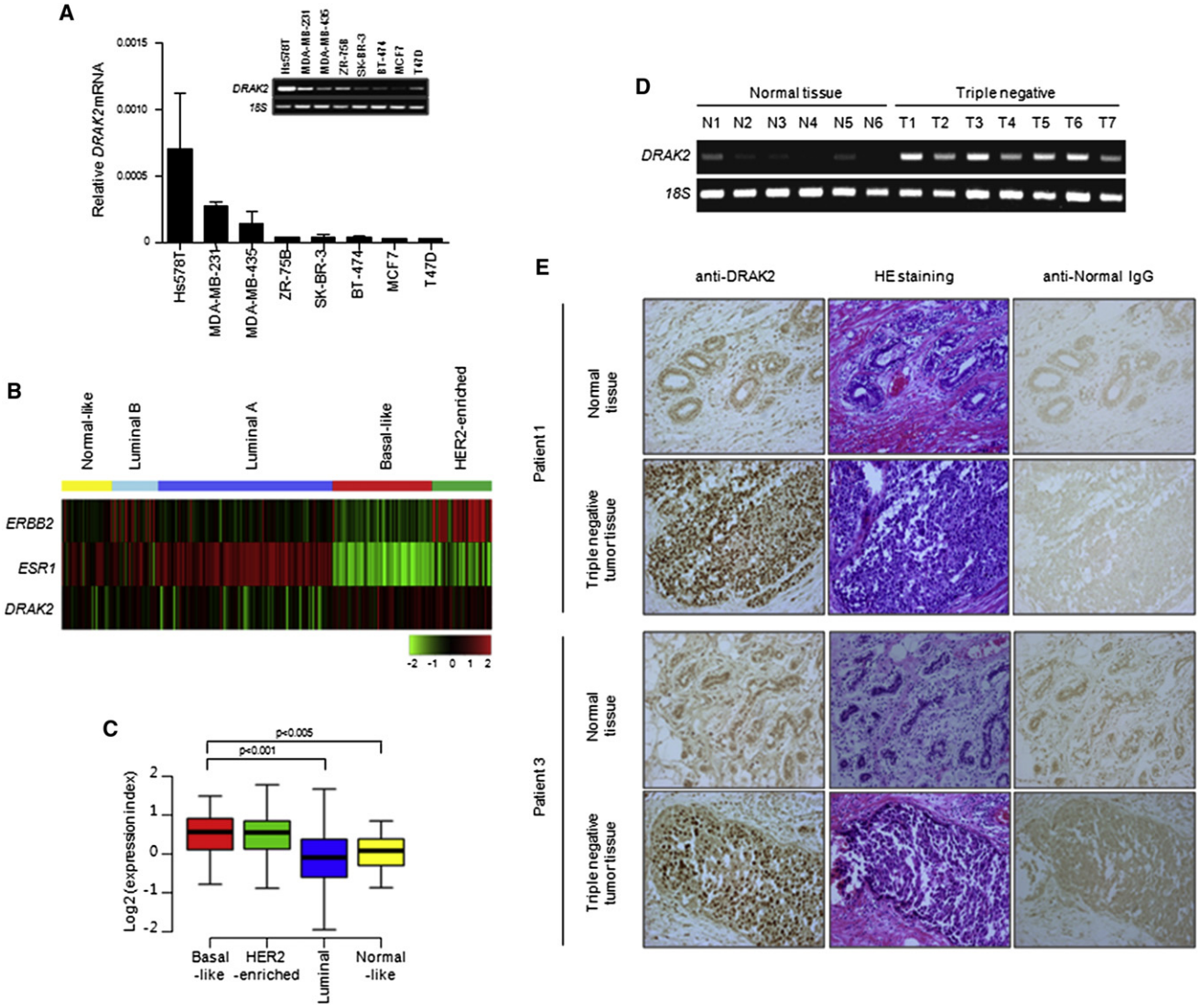

Figure 4. DRAK2 Expression Is Elevated in Primary Breast Cancers

(A) Quantitative RT-PCR of DRAK2 gene expression in various breast cancer cell lines.

(B) Heatmap of a published microarray data set showing the expression levels of *ERBB2*, *ESR1*, and *DRAK2* across the five breast cancer subtypes.

(C) Comparison of *DRAK2* expression in breast cancer subtypes using published microarray data sets.

(D) Expression level of DRAK2 mRNA was performed in six normal tissues and seven triple-negative breast tumor tissues using semiquantitative RT-PCR. 18S rRNA was used as an internal control.

(E) Histological analysis of normal tissue (top row) and tumor (bottom row) from two breast cancer patients. Left panels, staining with anti-DRAK2-specific antibody; middle panel, hematoxylin and eosin staining; right panel, staining with antinormal IgG antibody. Original magnification, ×200.

See also Figure S5.

observed the endogenous interaction between TβRI and DRAK2 even in the absence of TGF-β1, but TGF-β1 treatment increased the amount of DRAK2 associated with TβRI (Figure 5A). Interestingly, DRAK2 protein expression was enhanced upon TGF-β1 stimulation. The interaction of TβRI with DRAK2 also was confirmed in the in situ PLA. The number of DRAK2/TβRI complexes per cell was found to be significantly higher in the TGF-β1-treated cells (visualized as red dots in Figure 5B), suggesting that TGF-β1 enhances the association between DRAK2 and TβRI. To examine the role of DRAK2 in TGF-β1-induced transcription in Hs578T and MDA-MB-231 breast cancer cell lines, we examined the impact of DRAK2 expression on transcriptional activation of a TGF-β1-responsive reporter in these cell lines. As expected, ectopic expression of DRAK2 greatly decreased TGF-β1-induced transcriptional activity (Figures 5C and 5D). To determine if loss of DRAK2 expression enhances TGF-β signaling, we made Hs578T, MDA-MB-231, and HaCaT cell lines stably expressing *DRAK2*-specific small hairpin RNA (shRNA) using a lentiviral system. Inhibition of DRAK2 expression markedly enhanced TGF-β1-induced Smad2/3 phosphorylation in Hs578T

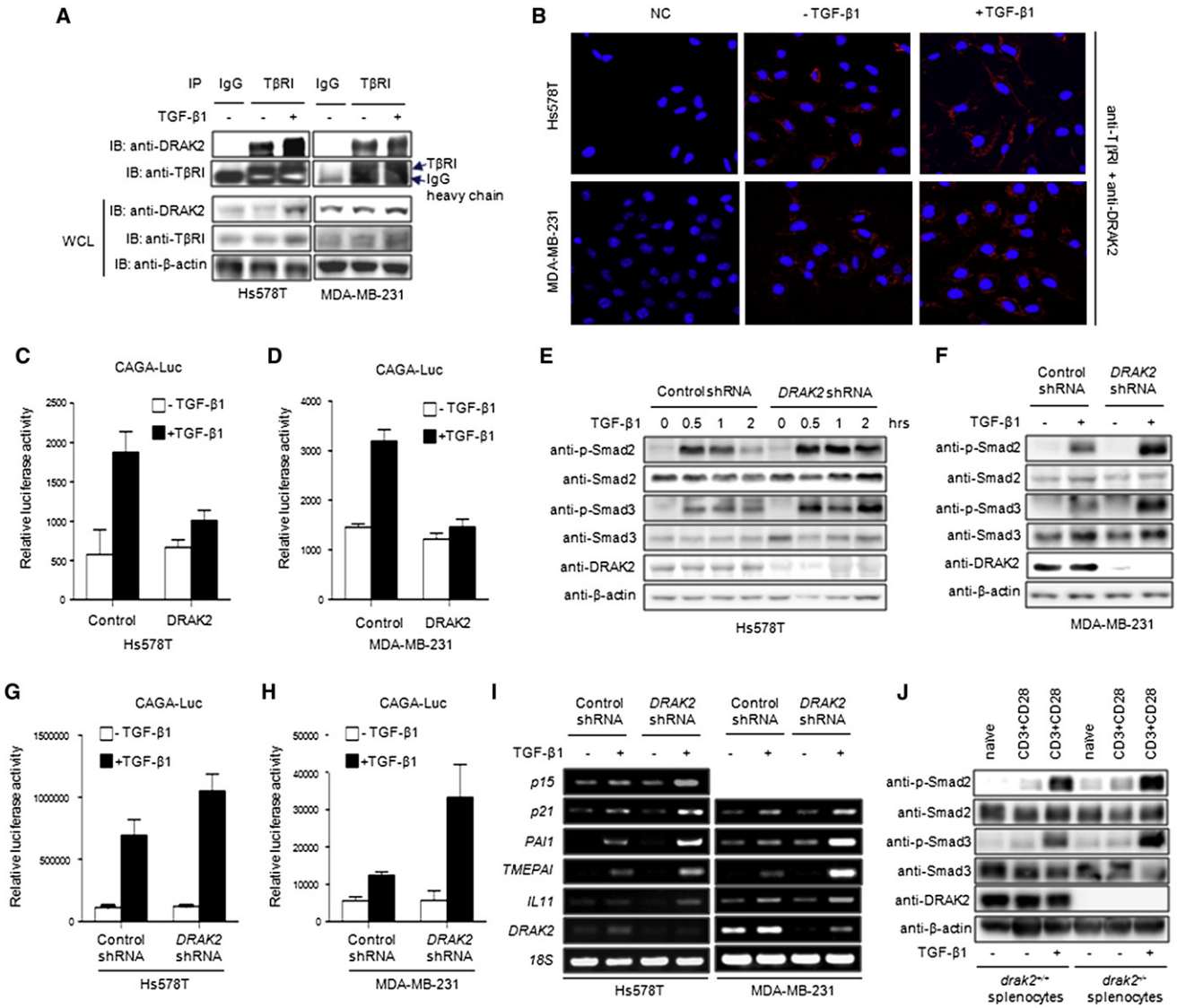

Figure 5. Knockdown of DRAK2 Increases TGF-β Signaling in Breast Cancer Cell Lines

(A) Hs578T and MDA-MB-231 breast cancer cell lines were treated with or without TGF-β1 for 4 hr, and cell lysates were immunoprecipitated with the antinormal IgG or anti-TβRI antibodies and then subjected to immunoblots with anti-DRAK2 antibody.

(B) Hs578T and MDA-MB-231 cells treated with TGF-β1 for 4 hr were fixed and assayed with in situ PLA. In situ PLA signals were visualized in red.

(C and D) Luciferase assays using lysates of Hs578T and MDA-MB-231 cells were performed by cotransfection of the TGF-β1-responsive reporter CAGA-Luc and Myc-tagged DRAK2, after incubation for 16 hr with or without TGF-β1 treatment. Error bars indicate SDM of three independent experiments.

(E) Immunoblot analysis of Hs578T cells stably expressing *DRAK2*-specific shRNA after TGF-β1 treatment for indicated times.

(F) Immunoblot analysis of MDA-MB-231 cells stably expressing *DRAK2*-specific shRNA after TGF-β1 treatment for 1 hr.

(G and H) Effect of DRAK2 knockdown on CAGA-Luc reporter activity to TGF-β1 in Hs578T and MDA-MB-231 cells stably expressing *DRAK2*-specific shRNA; TGF-β1 was added for 16 hr. Error bars indicate SDM of three independent experiments.

(I) Hs578T and MDA-MB-231 cells stably expressing *DRAK2*-specific shRNA were treated with TGF-β1 for 8 hr, and then total RNA was isolated. mRNA levels of TGF-β1 target genes, including *p15*, *p21*, *PAI1*, *TMEPAI*, *Il11*, *DRAK2*, and *18S*, were detected by RT-PCR.

(J) Splenocytes isolated from wild-type or *drak2*-knockout mice were stimulated by anti-CD3 and anti-CD28 antibodies for 2 hr. After TGF-β1 treatment for 1 hr, cells were collected and lysates were subjected to immunoblot analysis with indicated antibodies.

See also Figure S5.

and MDA-MB-231 breast cancer cells (Figures 5E and 5F) as well as HaCaT cells (Figure S6A). Next, we sought to examine whether inhibition of DRAK2 expression further enhances TGF-β1-induced transcriptional activity. Knockdown of DRAK2 significantly increased TGF-β1-induced transcriptional activities compared to the control (Figures 5G, 5H, and S6B). We also examined whether depletion of endogenous DRAK2 increases the expression of direct target genes of TGF-β signaling in

breast cancer cell lines. In comparison with the control cells, knockdown of DRAK2 increased the transcriptional level of the target genes in response to TGF-β1 in both cell lines (Figure 5I). To further confirm whether DRAK2 physiologically serves to inhibit TGF-β signaling in vivo, we investigated TGF-β1 responses in splenocytes isolated from *drak2* wild-type and *drak2*-deficient mice. Splenocytes from *drak2*-deficient mice showed much stronger induction of Smad2/3 phosphorylation by TGF-β1 treatment than splenocytes of *drak2* wild-type mice, consistent with previous results using shRNA-mediated knockdown (Figure 5J). We also found that Smad2/3 phosphorylation was reproducibly enhanced, even in the inactivated/unstimulated naive splenocytes of *drak2*-deficient mice.

TGF-β1 Regulates the Transcriptional Activation of DRAK2 Promoter

It is known that TGF-β signaling is subject to negative feedback through TGF-β1-induced transcripts such as Smad7 and TMEPAI. In the results shown above (Figures 2F, 5A, and 5I), we observed induction of expression of DRAK2 mRNA or protein by TGF-β1. Thus, we tested whether DRAK2 is a bona fide TGF-β1-responsive gene. DRAK2 expression was potently induced by TGF-β1 in many different cell lines: HeLa, HaCaT, Hs578T, and MDA-MB-231 cells (Figures 6A and S7A). We then examined whether TβRI kinase activity is required for the induction of DRAK2 expression by TGF-β1. TGF-β1 treatment induced expression of DRAK2 mRNA, whereas pretreatment of cells with a TβRI kinase inhibitor, SB431542, inhibited TGF-β1-induced DRAK2 mRNA expression (Figures 6B and S7B). In order to investigate whether TGF-β1-induced *DRAK2* expression required de novo protein synthesis, cells were pretreated with cycloheximide (CHX), a protein synthesis inhibitor. Expression of DRAK2 mRNA induced by TGF-β1 was increased in the presence of CHX, suggesting that DRAK2 was a direct target gene of TGF-β signaling (Figures 6C and S7C). Thus, DRAK2 is an early TGF-β1-responsive gene that acts in a negative feedback loop to suppress TGF-β signaling.

To determine whether expression of DRAK2 induced by TGF-β1 is associated with direct transcriptional activation of the DRAK2 promoter, a DNA fragment corresponding to the −1 to −4,775 bp region of the DRAK2 promoter was inserted into the upstream of a pGL3 luciferase reporter gene. This promoter fragment contained four putative Smad3-binding sites. When the DRAK2 promoter reporter construct was cotransfected with constitutively active TβRI (HA-Alk5 ca) into Hs578T cells, its promoter activity was increased by active TβRI in a dose-dependent manner (Figure 6D). TGF-β1 treatment also induced DRAK2 promoter activity, whereas its activity was blocked by treatment of TβRI kinase inhibitor (Figure 6E). To further characterize which Smad3-binding site is responsible for induction of DRAK2 expression by TGF-β1, four DRAK2 promoter deletion constructs were generated by deleting each putative Smad3-binding site. Deletions up to position −3,510 bp still maintained the responsiveness of DRAK2 promoter to constitutively active TβRI kinase, whereas deletion of position −3,100 to −3,510 bp abolished the transcriptional activity of DRAK2 promoter (Figure 6F). Thus, these findings support the existence of a functional TGF-β1-responsive element within the −3,510 to −4,755 bp region of the DRAK2 promoter. To examine whether TGF-β1 treatment triggers Smad3 binding to the DRAK2 gene chromatin, we performed chromatin immunoprecipitation (ChIP) using anti-Smad2/3 antibodies. The −3,035 to −3,359 bp promoter region interacted with Smad3 independent of TGF-β1 treatment, whereas TGF-β1 treatment significantly increased the interaction of Smad3 with the −4,481 to −4,833 bp promoter region. On the other hand, −2,404 to −2,752 and −4,111 to −4,488 bp regions did not interact with Smad3 (Figures 6G and S7D). To identify whether Smad3-binding sites located in −4,481 to −4,833 and −3,035 to −3,359 bp regions are important for TGF-β1-induced DRAK2 transcription, we generated mutant constructs for −4,481 to −4,833 and −3,035 to −3,359 bp regions (CAGA sequence replaced by GAAA). Luciferase activity driven by the full-length DRAK2 promoter was increased by constitutively active TβRI kinase; mutation of both putative Smad3-binding sites in −4,481 to −4,833 and −3,035 to −3,359 bp regions completely abolished the inducibility of DRAK2 promoter activity by constitutively active TβRI kinase (Figure 6H). Next, to further examine whether transcriptional activation of DRAK2 promoter by TGF-β1 is dependent on Smad3, we used a Smad3-deficient SNU484 gastric cancer cell to study Smad3 dependency on TGF-β1-induced DRAK2 promoter activity (Han et al., 2004). SNU484 cells lacking Smad3 did not respond to TGF-β1-dependent transcriptional activation of DRAK2 promoter, whereas ectopic expression of Smad3 rescued the DRAK2 promoter activity upon TGF-β1 treatment (Figure 6I). To exclude the possibility that the DRAK2 promoter is also regulated by Smad2, we performed the DRAK2 promoter assay using Smad2- or Smad3-deficient mouse immortalized mammary epithelial cells (IMECs) to see whether Smad2 activates DRAK2 promoter activity (Kohn et al., 2010). TGF-β1 induced DRAK2 promoter activity in wild-type IMECs as well as Smad2-deficient IMECs. However, TGF-β1 did not induce DRAK2 promoter activity in Smad3-deficient IMECs (Figure S7E). This result indicates that transcriptional activation of DRAK2 promoter by TGF-β1 is dependent on Smad3 rather than Smad2. Taken together, our results indicate that Smad3-binding elements in the DRAK2 promoter are responsible for transcriptional activation of DRAK2 by TGF-β1.

Knockdown of DRAK2 Significantly Suppresses Tumor Growth through Induction of TGF-β1-Mediated Tumor Suppressor Genes

TGF-β1-mediated cell growth inhibition occurs through induction of cell-cycle-arrest-related genes, such as $p15^{Ink4b}$ and $p21^{Waf1/Cip1}$. As shown in Figure 5I, knockdown of endogenous DRAK2 enhanced TGF-β1-induced expression of $p15^{INK4b}$ and $p21^{Waf1/Cip1}$ genes in Hs578T and MDA-MB-231 cells. Consistent with this observation, depletion of endogenous DRAK2 was antiproliferative in two breast cancer cell lines (Figures 7A and 7B). To examine whether knockdown of DRAK2 suppresses the transforming potential, Hs578T and MDA-MB-231 cells that stably express *DRAK2*-specific shRNA were seeded in soft agar plates. Knockdown of DRAK2 resulted in the decrease of anchorage-independent growth in soft agar compared to the control cells (Figures 7C and 7D). To confirm whether the loss

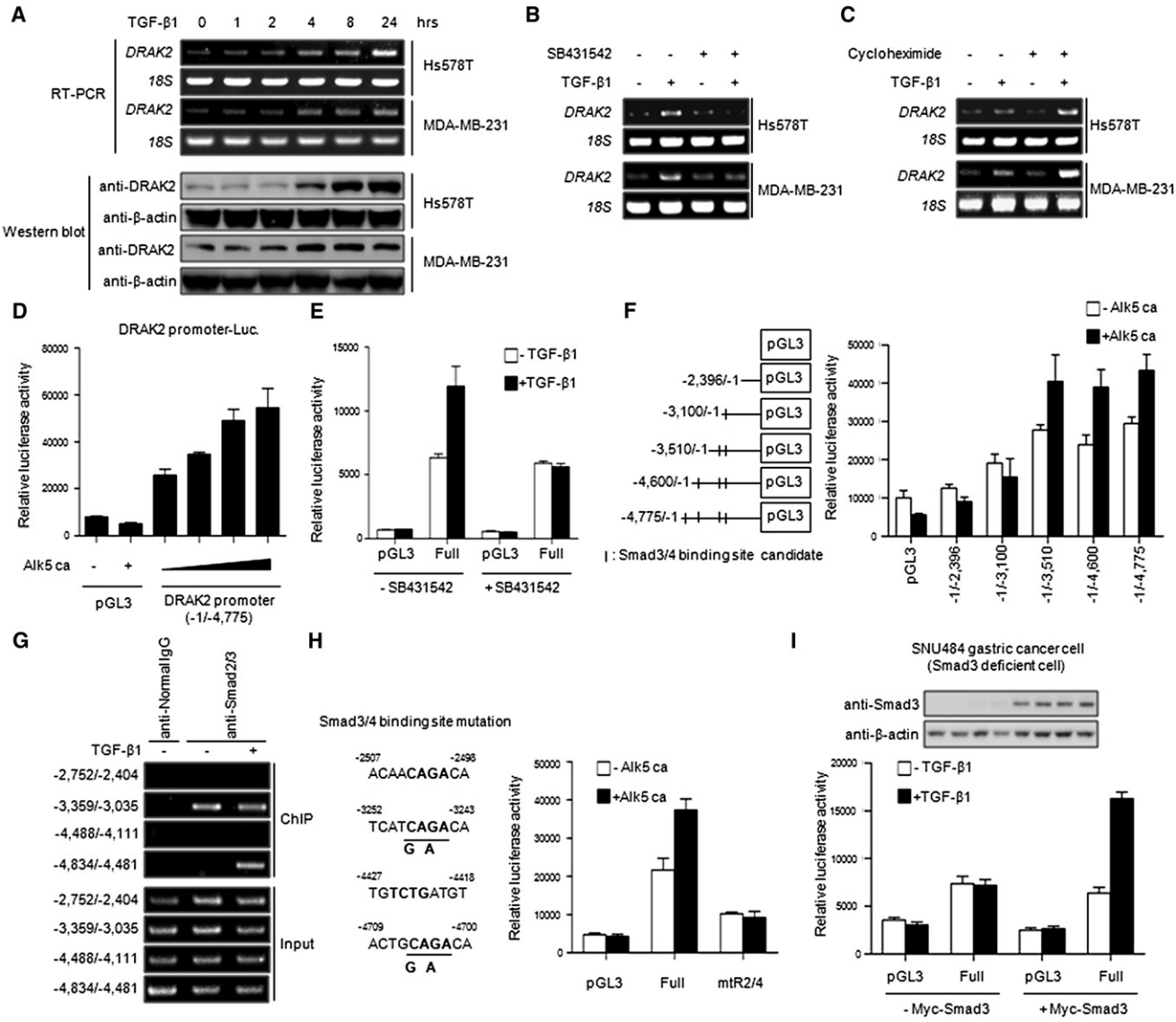

Figure 6. TGF-β1 Regulates the Transcriptional Status of the DRAK2 Promoter

(A) Hs578T and MDA-MB-231 cells were treated with TGF-β1 for indicated times and then RT-PCR (top) and immunoblots (bottom) were performed using a human DRAK2 primer and anti-DRAK2 antibody.

(B and C) Cells were stimulated with TGF-β1 for 4 hr after pretreatment with SB431542 (TβRI inhibitor) or cycloheximide for 2 hr. Total RNA was isolated and RT-PCR was carried out.

(D) Hs578T cells were transiently cotransfected with HA-tagged Alk5 ca, and pGL3 control or DRAK2 promoter including from −1 to −4,775 bp. Luciferase activities were normalized with β-galactosidase. Error bars indicate SDM of three independent experiments.

(E) Hs578T cells transfected with pGL3 control or DRAK2 full promoter were treated with or without TGF-β1 for 16 hr after pretreatment with SB431542 for 2 hr.

(F) Illustration of luciferase reporters including Smad3/4-binding site candidate regions in the DRAK2 promoter sequence (left). Hs578T cells were transfected with various promoter constructs and HA-tagged Alk5 ca and then treated with or without TGF-β1 for 16 hr. After TGF-β1 treatment, cells were assayed for luciferase activity. Error bars indicate SDM of three independent experiments.

(G) ChIP analysis showing the recruitment of Smad3 onto human DRAK2 promoter in the Hs578T cells treated with antinormal IgG or anti-DRAK2 antibodies in the presence or absence of TGF-β1 treatment for 8 hr.

(H) The consensus Smad3-binding sites are underlined and their mutations are shown in lowercase characters (left). Hs578T cells were cotransfected with the HA-tagged Alk5 ca and pGL3 control, DRAK2 full promoter, or double-mutant reporter containing mutated region II and region IV (CAGA sequence replaced by GAAA) Smad3 sites and then subjected to luciferase assays. Error bars indicate SDM of three independent experiments.

(I) Smad3-deficient SNU484 gastric cancer cells were cotransfected with Flag-tagged Smad3 and pGL2 control or DRAK2 full promoter sequence reporter and then treated with or without TGF-β1 for 16 hr. Cell lysates were collected for luciferase assays. Error bars indicate SDM of three independent experiments.

See also Figure S7.

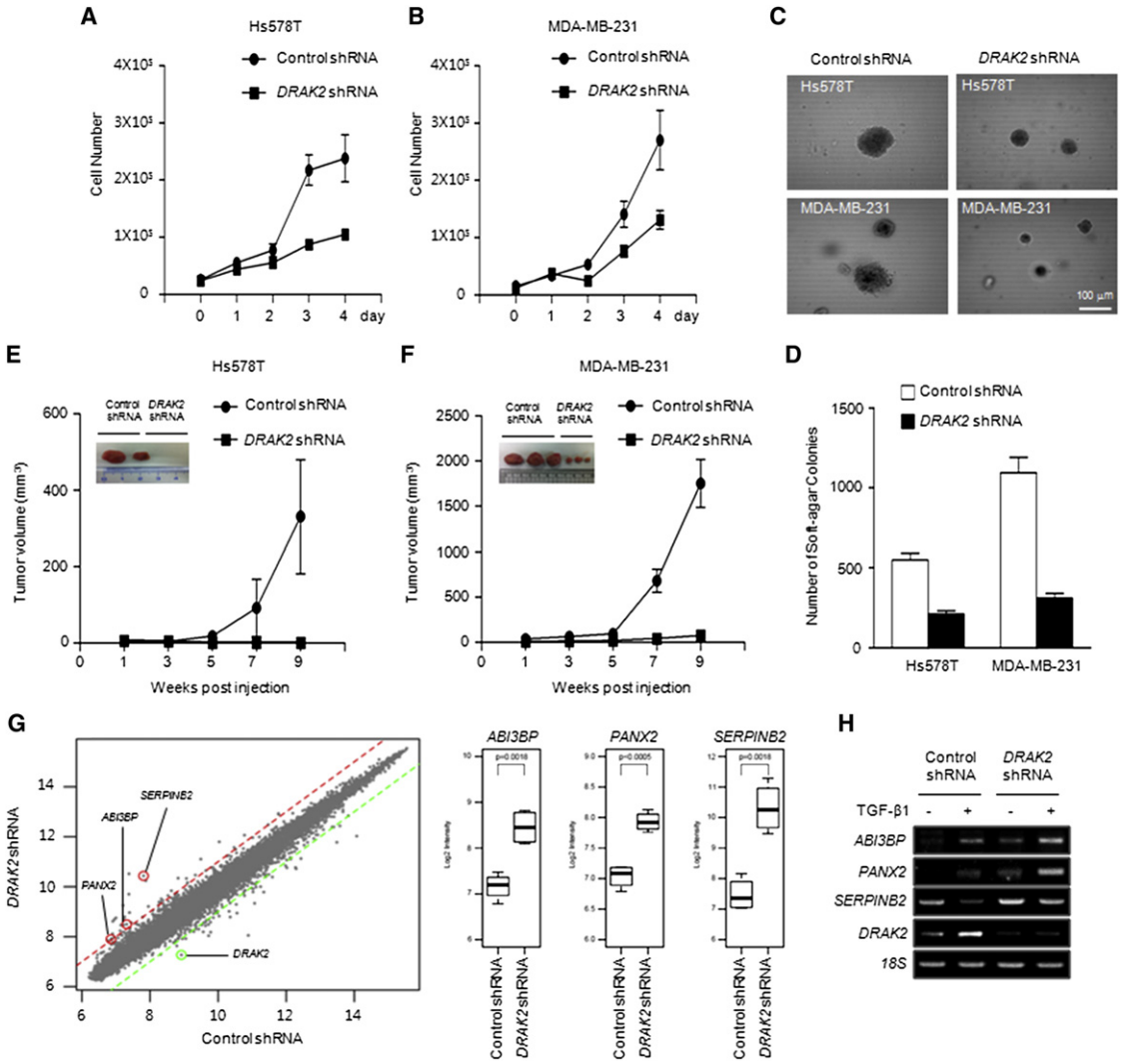

Figure 7. Knockdown of DRAK2 Significantly Inhibits Tumor Growth through Induction of TGF-β1-Mediated Tumor Suppressor Genes

(A and B) Cell doublings of Hs578T and MDA-MB-231 stably expressing *DRAK2*-specific shRNA. Each data point represents the mean of cells counted in triplicate dishes.

(C) Anchorage-independent growth of Hs578T and MDA-MB-231 stably expressing *DRAK2*-specific shRNA. Photographs of colony formation are shown as microscopic colonies formed at 3 weeks after seeding.

(D) The number of crystal violet-stained colonies presented is the mean of colony counts in ×50 microscopic fields from three dishes. Error bars indicate SDM of three independent experiments.

(E and F) Tumor formation and volumes of Hs578T and MDA-MB-231 cells stably expressing *DRAK2*-specific shRNA subcutaneously injected into SCID mice. Error bars indicate SDM of three independent experiments.

(G) Scatter plot comparing global gene expression profiles between the *DRAK2* shRNA and control shRNA Hs578T cell lines. Red and green lines indicate 2-fold differences in either direction of gene expression levels. Boxplot comparing validated gene expression profiles between the *DRAK2* shRNA and control shRNA in Hs578T cell lines was also shown.

(H) RT-PCR validation of genes upregulated in the Hs578T cells stably expressing *DRAK2*-specific shRNA. Hs578T cells stably expressing control or *DRAK2*-specific shRNA were treated with or without TGF-β1 for 16 hr and then subjected to RT-PCR.

of DRAK2 expression impacted the tumorigenic capacity in breast cancer cells in vivo, we tested the tumorigenicity of Hs578T and MDA-MB-231 cells that stably express either *DRAK2*-specific shRNA or control shRNA in immunodeficient mice. Knockdown of DRAK2 decreased the ability of Hs578T and MDA-MB-231 cells to form tumors, suggesting that DRAK2 was required to reinforce tumorigenicity in vivo (Figures 7E and 7F). To confirm that reduced tumorigenic potential causes the induction of tumor suppressor genes, we performed microarray analyses in Hs578T cells that stably express *DRAK2*-specific shRNA. Microarray analysis revealed that knockdown of DRAK2 induced changes in the gene expression profile. Among the differentially expressed genes that might be involved in tumor suppression, *ABI3BP* (ABI gene family member 3-binding

protein), *PANX2* (pannexin2), and *SERPINB2* (PAI-2) were strongly upregulated in cells bearing *DRAK2*-specific shRNA (Figure 7G). Next, the expression of these genes was validated by RT-PCR. Knockdown of DRAK2 increased basal expression of these genes and expression of PANX2 and ABI3BP genes was strongly induced by TGF-β1 (Figure 7H). Taken together, these results suggest that DRAK2 may promote tumorigenesis by downregulating expression of tumor suppressor genes, in part, through suppression of TGF-β1 tumor suppressor activity.

DISCUSSION

It is well known that the TGF-β signal transduction pathway is tightly controlled by various positive and negative regulatory nodes. Each step in the TGF-β signaling pathway is under intense regulation to maintain a balance between positive and negative control. Dysregulation of this pathway can lead to various diseases, including inflammatory diseases as well as cancer development (Massagué et al., 2000; Massagué, 2008). Our results here indicate that DRAK2 serves as a TGF-β1-inducible molecule that plays a role in terminating TGF-β signaling. DRAK2 directly binds to TβRI and this interaction prevents Smad2 and Smad3 from active participation in TGF-β signaling. Because TGF-β1 rapidly induces expression of DRAK2 mRNA, it is likely that DRAK2 may participate in a negative feedback loop to control TGF-β1 responses and control the intensity and duration of TGF-β signaling. Our findings provide evidence for a unique role of DRAK2 as a negative regulator of TGF-β signaling that shows immediately early response in the TGF-β signaling pathway.

Several proteins, including Smad7 and TMEPAI, which are themselves TGF-β1 target genes, have been reported to play negative roles in the regulation of TGF-β signaling by either preventing interaction of R-Smads with TβRI or inhibiting activation of R-Smads. Previous studies have shown that Smad7 associates stably with the activated TβRI to inhibit TGF-β signaling by preventing access of Smad2/3 to the receptor kinase domain (Hayashi et al., 1997). Smad7 also recruits the HECT type of E3 ubiquitin ligases, Smurf1/2, and leads to the degradation of TβRI through the proteasomal pathway (Kavsak et al., 2000; Ebisawa et al., 2001). TMEPAI is known to antagonize TGF-β1-induced R-Smads phosphorylation by competing with SARA for binding to R-Smads (Watanabe et al., 2010). Unlike Smad7 or TMEPAI, DRAK2 does not induce the degradation of TβRI or compete with SARA for the interaction with receptors. Instead, DRAK2 represents a naturally occurring receptor kinase antagonist that functions to inhibit TGF-β signaling through direct physical interaction with TβRI, thereby blocking the interaction between activated TβRI and R-Smads. Interestingly, DRAK2 interacts with TβRI regardless of the activation status of TβRI, while Smad7 only interacts with the activated TβRI. This finding suggests that DRAK2 may be a general inhibitor that functions at a very early step in the TGF-β signaling pathway. To support this, we confirmed that overexpression of DRAK2 suppressed TGF-β1-induced *TMEPAI* expression, whereas depletion of DRAK2 by specific shRNA significantly enhanced its expression. Thus, it is likely that DRAK2 constitutively restricts TβRI kinase activity to block its downstream signaling. These conclusions are consistent with the general suppression that we observed in all of the transcriptional reporter assays, and in target gene expression assays in cells either overexpressing DRAK2 or reducing *DRAK2* expression by specific shRNA employed to study TGF-β signaling.

DRAK2 kinase activity is regulated in a calcium-dependent manner. DRAK2 is phosphorylated on at least two distinct sites, Ser^{10} and Ser^{12}. The Ser^{12} site for autophosphorylation has been shown to play key roles in modulating the function of DRAK2. The earlier study demonstrated that Ser^{12} phosphorylation is necessary for optimal suppression of T cell activation by this kinase (Friedrich et al., 2007). However, Ser^{12} mutation did not interfere with kinase activity. We also found that the S12A mutant retained the inhibitory activity of TGF-β signaling (data not shown). Most surprisingly, the kinase-inactive mutant of DRAK2 was almost completely inactive in suppressing TGF-β signaling, and its binding activity to TβRI was markedly reduced (Figure 3). Based on work presented here, we have shown that proper dimerization is critical in enhancing the binding affinity to TβRI leading to the suppression of TGF-β signaling. In this case, the proper homodimerization of DRAK2 can occur only when each DRAK2 monomer is catalytically active. This suggests that monomer autophosphorylation may be required for homodimerization. DRAK2 dimerization may affect the binding to TβRI by imposing, for example, via a conformational change in the structure of DRAK2, thereby facilitating interactions with TβRI. Further analysis of DRAK2 phosphorylation and dimerization events, combined with molecular modeling of the catalytic domain, will establish the novel mode of action of this kinase.

We demonstrated that the transcriptional regulation of DRAK2 by TGF-β1 is mediated through a rapid and direct Smad3-dependent signaling mechanism. TGF-β1 treatment rapidly induces DRAK2 expression, resulting in increased interactions between endogenous TβRI and DRAK2. This interaction was found to occur in the cytoplasm and near the plasma membrane. The ability of the DRAK2 promoter to respond to TGF-β1 is therefore believed to play a central role in the negative autoregulation of TGF-β signaling. However, they do not rule out the possibility that this protein kinase may fulfill other functions in normal physiology. In addition to suppressing TGF-β signaling within the cytosol, DRAK2 is predominantly localized in the nucleus in the breast cancer cells analyzed, even in the presence of TGF-β1. Although this has not yet been directly investigated, a possible nuclear role for DRAK2 may be in the regulation of transcription. This observation is supported by the microarray analysis of genes regulated by DRAK2. Furthermore, DRAK2 is known to be induced by combinatory network of proinflammatory cytokines, such as IFN-γ with IL-1β or TNF-α with IL-1β (Mao et al., 2009). Thus, DRAK2 not only is a TGF-β1-induced antagonist but is also involved in fine-tuning the cellular response to TGF-β1, by integrating different signaling pathways.

All members of the DAPK family have been shown to induce apoptosis upon ectopic expression in various cell types (Köqel et al., 2001). However, the role of DRAK2 in apoptosis has been controversial. Its ability to induce apoptosis seems to depend on the level of expression, the cell types studied, and the intracellular localization of the kinase. Upregulation of DRAK2 by inflammatory stimuli results in increased apoptosis

of islet β cells and T cells (Mao et al., 2006, 2009). In contrast, other studies have reported that *drak2*-deficient mice did not show any defects in T cell apoptosis, suggesting that DRAK2 may function in maintaining the survival of proliferating T cells (McGargill et al., 2004; Ramos et al., 2008). Moreover, analysis of potential tumor-associated genes expressed in cutaneous T cell lymphoma (CTCL) revealed enhanced DRAK2 expression in the sera or tumor tissues of CTCL patients, but not in healthy donors (Hartmann et al., 2008).

In numerous tumors, the disruption of TGF-β signaling has shown to be a critical step in the development of tumorigenic potential. The underlying mechanism of resistance to the growth inhibitory effect of TGF-β1 in malignant cells involves the altered expression as well as inactivating mutations of either receptors or components of the signaling pathways such as Smad3 or Smad4. The fact that the DRAK2 protein functions as an antagonist of TGF-β signaling suggests that amplified expression of this protein in human breast cancers might promote suppression of TGF-β signals in tumors. Indeed, our studies demonstrated that the elevation of DRAK2 expression contributes to the progression of human breast cancer. Depletion of DRAK2 markedly decreased the proliferation of breast cancer cell lines and suppressed tumorigenic capacity. Further supporting this hypothesis, overexpression of DRAK2 is observed in basal-like and HER2-enriched breast tumors compared to normal and luminal cell types. Future studies will provide a deeper insight into the role of this pathway during tumorigenesis.

In conclusion, we have demonstrated that DRAK2 is a negative regulator of TGF-β signaling, inhibiting the phosphorylation of R-Smads through its interaction with TβRI. Our findings presented here implicate a function of DRAK2 in the limiting of TGF-β1 growth inhibitory activity. Considering the evidence that aberrant expression of components related to TGF-β signaling is strongly linked to tumorigenicity in breast cancer, DRAK2 may become a potential therapeutic target for this disease.

EXPERIMENTAL PROCEDURES

Materials

Cell cultures, reagents, antibodies, and transfections are described in detail in the Extended Experimental Procedures.

Lentiviral shRNAs for DRAK2

DRAK2 shRNA (TRC781) lentiviral vectors (Sigma-Aldrich) were used for knockdown of DRAK2.

Immunoprecipitation and Immunoblot Analysis

Trasnfected or untransfected cells were washed twice in cold PBS and lysed in IP buffer (50 mM Tris [pH 7.4], 150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 2 mM EDTA, 10% glycerol) plus phosphatase and protease inhibitors (Roche). Whole-cell extracts were incubated with the appropriate primary antibodies overnight at 4°C. Antibody-bound proteins were precipitated with protein A/G beads according to the protocol provided by the manufacturer. After the beads were washed three times with lysis buffer, they were eluted in 2 × SDS sample loading buffer. Eluted proteins were separated by SDS-PAGE and then detected by immunoblots.

In Situ Proximity Ligation Assay

In situ PLA was performed according to the manufacturer's protocol (Olink Bioscience). Cells were immediately fixed in 4% paraformaldehyde in 4°C for 30 min. Slides were blocked, incubated with antibodies directed against Myc, HA, TβRI, and DRAK2 and then incubated with PLA PLUS and MINUS probe for anti-mouse and anti-rabbit, which secondary antibodies conjugated to unique oligonucleotides. Slides were mounted with Vectashield mounting media (Vector Laboratories), and immunofluorescence images were obtained by the confocal microscope.

Chromatin Immunoprecipitation

ChIPs were carried out according to the Millipore protocol. The detail procedures were described in the Extended Experimental Procedures.

Tumorigenicity

Hs578T and MDA-MB-231 breast cancer cell lines stably expressing control vector or *DRAK2*-specific shRNA (5×10^6) were injected subcutaneously into the flanks of SCID mice. Tumor dimensions were measured twice weekly. Mice were sacrificed at 9 weeks after injection, and tumors were surgically isolated. Tumor volume (V) was calculated by using the formula $(S \times S \times L) \times 0.5$, where S and L were the short and long dimensions, respectively. All animals were maintained according to the CHA Hospital Animal Care and Use Committee guidelines under protocol number IACUC110004.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, seven figures, and one table and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2012.09.028.

LICENSING INFORMATION

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