ELL Protein-associated Factor 2 (EAF2) Inhibits Transforming Growth Factor β Signaling through a Direct Interaction with Smad3*

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Background: EAF2 plays an important role in affecting cellular processes, but the mechanisms underlying its effects are poorly understood.

Results: EAF2 was identified as a novel repressor of TGF-β signaling through a direct interaction with Smad3.

Conclusion: EAF2 specifically interacts with Smad3 to prevent Smad3 nuclear translocation, Smad4-Smad3 complex formation, and p300-Smad3 complex formation.

Significance: EAF2 may function by inhibiting TGF-β signaling.

A series of in vitro and in vivo studies has shown that EAF2 can affect multiple signaling pathways involved in cellular processes. However, the molecular mechanisms underlying its effects have remained elusive. Here we report the discovery of a new functional link between EAF2 and TGF-β signaling. Promoter reporter assays indicated that EAF2 suppresses Smad3 transcriptional activity, resulting in inhibition of TGF-β signaling. Coimmunoprecipitation assays showed that EAF2 specifically interacts with Smad3 in vitro and in vivo but not with other Smad proteins. In addition, we observed that EAF2 binding does not alter Smad3 phosphorylation but causes Smad3 cytoplasmic retention, competes with Smad4 for binding to Smad3, and prevents p300-Smad3 complex formation. Furthermore, we demonstrated that EAF2 suppresses both TGF-β-induced G1 cell cycle arrest and TGF-β-induced cell migration. This study identifies and characterizes a novel repressor of TGF-β signaling.

However, the molecular mechanisms underlying EAF2 functions remain largely unknown.

Transforming growth factor β (TGF-β) signaling plays important roles during embryogenesis and in the regulation of cell functions and in tissue homeostasis (8–10). TGF-β signaling is activated via a large family of structurally related cytokines including TGF-β, activins, and bone morphogenetic proteins through binding to heterotetrameric complexes of type I and type II serine/threonine kinase receptors (8, 9, 11). Smad proteins are critical mediators of TGF-β signaling. After stimulation by TGF-β, the constitutively active type II receptor phosphorylates and activates the type I receptor. Subsequently, the members of the Smad family known as the receptor-activated Smads (R-Smads), Smad2 and Smad3, are activated by type I receptor-mediated phosphorylation. The phosphorylated R-Smads form complexes with Smad4 that translocate to the nucleus and regulate the transcription of specific genes in cooperation with other transcription factors, co-activators, and co-repressors (10). In order to perform their crucial roles in TGF-β signaling, Smad proteins are regulated by post-translational modifications such as phosphorylation, ubiquitination, and acetylation (10).

Given that EAF2 negatively regulates two important signaling pathways involved in many aspects of cell functionality, canonical Wnt/β-catenin signaling, and hypoxia signaling pathways (3, 4, 6, 7), we attempted to determine whether EAF2 also influences TGF-β signaling. In this study we found that EAF2 attenuates TGF-β signaling and specifically interacts with Smad3 in vitro and in vivo but not with other Smad proteins. Moreover, we found that EAF2 impairs Smad3 nuclear translocation, disrupts Smad3-Smad4 complex formation, and prevents the binding of p300 to Smad3. Consequently, EAF2 attenuates TGF-β-induced G1 cell cycle arrest and cell migration. Therefore, EAF2 plays an important role in affecting cellular processes, but the mechanisms underlying its effects are poorly understood.

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2 The abbreviations used are: R-Smad, receptor-activated Smad; SARS, severe acute respiratory syndrome; CBP, CREB-binding protein; MEF, mouse embryonic fibroblasts.
fore, our work describes a newly identified player in the TGF-β signaling pathway.

### Experimental Procedures

**Cell Line and Culture Conditions**—HEK293T, HepG2, and MDA-MB-231 cells were originally obtained from ATCC. HaCaT and Mv1Lu cells were kindly provided by Dr. Ye-Guang Chen. HEK293T and HepG2 cell lines were cultured in DMEM (HyClone) with 10% fetal bovine serum (FBS). MDA-MB-231 cells were cultured in L15 (HyClone) with 10% FBS. EAF2 wild type and null MEF cells, and HaCaT cells were maintained in DMEM supplemented with sodium pyruvate, 10% FBS, 0.1 mmol/liter non-essential amino acids (Sigma) and 1% penicillin/streptomycin. Mv1Lu cells were cultured in minimum essential medium (HyClone) with 10% FBS. All cells were grown at 37 °C in a humidified incubator containing 5% CO₂.

**Plasmid Constructions**—The SBE4-Luc reporter, ARE-Luc reporter, and HA-Smad4 expression plasmid were provided by Dr. Xin-Hua Feng. Human Smad3 domains were subcloned into pCMV-Myc (Clontech), pCMV-HA (Clontech), and pCMV-Tag2B (Stratagene) vectors. Human Smad3 domains were PCR-amplified and subcloned into the pCMV-Myc (Clontech) or pCMV-Tag2B (Stratagene) vector. Human Smad3 domains were subcloned into pGEX-2T, and the human EAF2 gene was cloned into pET-32α (Novagen). Human EAF2 domains were subcloned into pCMV-Myc vector. EAF2-shRNAs and control-shRNA (GFP) were described previously (7). Human Smad3 gene was subcloned into pM vector (Clontech).

**Antibodies and Chemical Reagents**—Anti-EAF2 antibody was raised by injecting GST-EAF2 into rabbits or purchased from Epitomics. Anti-Myc (9E10), anti-GAPDH (sc-47724), anti-p21 (C-19), anti-His (H-15), anti-p300 (F-4), and anti-Smad4 (B-8) antibodies were purchased from Santa Cruz. Anti-HA antibody was purchased from Covance. Anti-FLAG (F1804) antibody was purchased from Sigma. Anti-α-tubulin (EPR1333), anti-Smad2 (EP784Y), anti-Smad3 (EP568Y), and anti-Smad3 (Ser(P)-423/425) (F1804) antibody was purchased from Epitomics. Anti-PAI-1 (612025) antibody was purchased from Sigma. Anti-HA antibody was purchased from Covance. Anti-FLAG (F1804) antibody was purchased from Sigma. Anti-α-tubulin (EPR1333), anti-Smad2 (EP784Y), anti-Smad3 (EP568Y), and anti-Smad3 (Ser(P)-423/425) (F1804) antibody was purchased from Epitomics. Anti-PAI-1 (612025) antibody was purchased from BD Transduction Laboratories. Recombinant human TGF-β1 was purchased from R&D Systems. Recombinant human bone morphogenetic protein-2 was purchased from Peprotech.

**Transfection Reagents**—Due to cell death induction in HaCaT cell by VigoFect (Vigorous Biotechnology, Beijing, China), FuGENE 6 (Roche Applied Science) was chosen for HaCaT cell transfection; other cells were transfected with VigoFect.

**Luciferase Reporter Assays**—Cells were seeded in 24-well plates and transfected with the indicated luciferase reporters using VigoFect (Vigorous Biotech). pTK-Renilla was used as an internal control. For mammalian one-hybridization assays, the pFR-luciferase construct (Stratagene) was used as a reporter. After transfection, the cells were treated with TGF-β (2 ng/ml) or control for 18 h. Luciferase activity was measured by the Dual-luciferase Reporter Assay System (Promega). The data are reported as the mean ± S.E. of three independent experiments performed in triplicate. The statistical analysis was performed using GraphPad Prism 5 (unpaired t test) (GraphPad Software Inc.).

**Co-immunoprecipitation and Western Blot Analysis**—Antic-Myc antibody and anti-HA antibody-conjugated agarose beads were purchased from Sigma. Protein A/G-Sepharose beads were purchased from GE Healthcare. Glutathione S-transferase (GST)-Bind resin was purchased from Novagen. For Western blot analysis and co-immunoprecipitation of over-expressed proteins, the experimental procedures have been described previously (5). For endogenous co-immunoprecipitation, the experimental procedures have been described previously (7). For GST pulldown assays, GST-tagged Smad3 and His-tagged EAF2 were expressed in Escherichia coli (BL21) and purified. After co-immunoprecipitation using GST-Bind resin, the protein was separated by SDS-PAGE. The gel was stained with Coomassie Blue or transferred to a PVDF membrane for detecting His-EAF2 by Western blot analysis. The Fuji Film LAS4000 mini luminescent image analyzer was used for photographing the blots. Multi Gauge V3.0 was used for quantifying the protein levels based on the band density obtained in Western blot analysis.

**Semi-quantitative Real Time PCR**—The total RNA was extracted by TRIzol reagent (Invitrogen). The cDNA synthesis was carried out using first-strand cDNA synthesis kit (Fermentas). The primers were synthesized following the previous report (14).

**Nucleus and Cytoplasm Separation**—Nucleus and cytoplasm separation was performed using the Nucl-Cyto-Mem Preparation kit (P1200, APPLYGEN). Briefly, HaCaT cells were transfected with the indicated plasmids by FuGENE 6 following the manufacturer’s protocol. After transfection for 24 h, the cells were treated with vehicle control (PBS) or TGF-β (2 ng/ml) for 2 h. Then the cells were washed with PBS and homogenized with a syringe for 40 times in 500 μl of CER buffer provided by the kit. The homogenate was centrifuged at 800 × g for 5 min, and the pellet was saved as the crude nuclei, which was then washed twice with 500 μl of NER buffer provided by the kit. The protein levels were detected by Western blot.

**Cell Cycle Analysis**—HeCaT cells were infected with EAF2 or control lentivirus and treated with TGF-β (2 ng/ml) for 20 h followed by FACS analysis.

**Cell Growth Assays**—MEF cells were seeded in 6-well plates at 3 × 10⁴ cells/well. TGF-β (2 ng/ml) was added to cells. After 24 h, the cells were counted by TC-20 cell counter (Bio-Rad).

**Cell Migration Assays**—MEF cells in the transwell were treated with TGF-β (200 pm) for 16 h and fixed with methanol. After Giemsa staining, the migrated cells were photographed and quantitated.

**Immunohistochemical Staining**—Human prostate tissue sections were deparaffinized using xylene and rehydrated in graded ethanol (EtOH) series. Rabbit monoclonal anti-EAF2 (Epitomics, 7118-1) and anti-Smad3 (Epitomics, EP568Y) were used for staining. Hematoxylin and eosin were used for counterstaining.
Results

**EAF2 Attenuates TGF-β Signaling**—To understand the mechanism of EAF2 action in affecting cell function, we sought to determine whether EAF2 could affect TGF-β signaling. Initially, we performed promoter assays by taking advantage of TGF-β-responsive luciferase reporters, including CAGA luciferase reporter, SBE4 luciferase reporter, and ARE luciferase reporter (12, 13). Upon stimulation with TGF-β (2 ng/ml), the activity of the CAGA luciferase reporter was much lower in EAF2 wild type MEF cells (EAF2+/+) compared to that in EAF2 null MEF cells (EAF2−/−) (p = 0.0251). Overexpression of EAF2 in Mv1Lu cells resulted in dramatic decreases in CAGA luciferase reporter activity. C, overexpression of EAF2 in Mv1Lu cells reduced SBE4 luciferase reporter activity in the presence of TGF-β (2 ng/ml) (p = 0.0012). D, overexpression of EAF2 in HepG2 cells did not alter ARE luciferase reporter activity. E, knockdown of EAF2 by EAF2-shRNAs (EAF2-shRNA-1 and EAF2-shRNA-2) in HaCaT cells enhanced CAGA luciferase reporter activity. The efficiencies of EAF2-shRNA-1- and EAF2-shRNA-2-mediated knockdown of EAF2 were confirmed. F, knockdown of EAF2 by EAF2-shRNAs (EAF2-shRNA-1 and EAF2-shRNA-2) in HaCaT cells enhanced SBE4 luciferase reporter activity. The efficiencies of EAF2-shRNA-1- and EAF2-shRNA-2-mediated knockdown of EAF2 were confirmed. The cells were transfected with the indicated plasmids; after 18–24 h, the cells were harvested for luciferase assays. Data are presented as the mean ± S.E. of three independent experiments performed in triplicate.
activity after the addition of TGF-β (2 ng/ml) (Fig. 1C). Interestingly, overexpression of EAF2 had no effect on ARE luciferase reporter activity in HepG2 cells (Fig. 1D). The ARE-luciferase reporter is known to be activated by both TGF-β and activin via Smad2, Smad4, and FoxH (18), implying that Smad2, Smad4, and FoxH might not be the mediators of EAF2 in TGF-β signaling. In contrast, knockdown of EAF2 by shRNAs (EAF2-shRNA-1 and EAF2-shRNA-2) in HaCaT cells enhanced the protein levels of p21 in the presence of TGF-β (2 ng/ml) (Fig. 1, E and F). The efficiencies of both EAF2-shRNA-1- and EAF2-shRNA-2-mediated knockdown of EAF2 in HaCaT cells were confirmed using Western blot analysis (Fig. 1, E and F). Therefore, all of these results indicate that EAF2 might inhibit TGF-β signaling.

To further determine whether EAF2 indeed attenuates TGF-β signaling, we examined the expression of the downstream target genes of TGF-β signaling using semiquantitative RT-PCR assays and Western blot assays. Upon TGF-β stimulation (2 ng/ml), the expression of Ctgf, Cxcr4, Pai-1, and Smad7 in EAF2 wild-type MEF cells (EAF2+/+) were much lower than their observed expression in EAF2-null MEF cells (EAF2−/−) (Fig. 2A) (14). Moreover, overexpression of EAF2 in HaCaT cells caused expression of PAI-1 and MMP9 to dramatically decrease in the presence of TGF-β (2 ng/ml) (Fig. 2B). Overexpression of EAF2 in HaCaT cells also caused a decrease of PAI-1 and p21 protein levels in the presence of TGF-β (Fig. 2C). In contrast, knockdown of EAF2 using shRNAs (EAF2-shRNA-1 and EAF2-shRNA-2) in HaCaT cells caused an increase of p21 protein levels in the presence of TGF-β (Fig. 2C). Taken together, these results suggest that EAF2 inhibits TGF-β signaling; however, this inhibition is probably not mediated through EAF2 interactions with Smad2, Smad4, and FoxH.

**EAF2 Interacts with Smad3**—Due to the key regulatory role of the Smad protein family in TGF-β signaling (10) and to gain insight into the mechanism by which EAF2 inhibits TGF-β signaling, we examined whether EAF2 interacts with Smad proteins. Initially, we performed coimmunoprecipitation assays by co-transfecting HEK293T cells with Myc-tagged EAF2 together with FLAG-tagged or HA-tagged Smad1, Smad2, and Smad4. As shown in Fig. 3A, EAF2 only interacted with Smad3, which is consistent with the above observations that EAF2 has no effect on ARE luciferase reporter activity. To further confirm that EAF2 only interacts with Smad3, we used HA-tagged Smad1, Smad2, and Smad3 to pull down Myc-tagged EAF2. The expression levels of HA-tagged Smad1, Smad2, and Smad3 were quite equivalent, but only HA-tagged Smad3 co-immunoprecipitated with Myc-EAF2 (Fig. 3B). In addition, we found that Smad7 also could not associate with EAF2 (Fig. 3C).

To examine whether EAF2 and Smad3 proteins interact endogenously, we performed coimmunoprecipitation assays using HaCaT cell extracts by an anti-Smad3 antibody and found that Smad3 interacted with endogenous EAF2 in the
EAF2 Inhibits Smad3 Transcriptional Activity—Because EAF2 inhibits TGF-β signaling and specifically interacts with Smad3, we sought to determine whether EAF2 could inhibit Smad3 transcriptional activity. In Mv1Lu cells, overexpression of EAF2 in incrementally increasing doses did not alter Smad3 protein levels (Fig. 6A). However, overexpression of EAF2 in incrementally increasing doses did not alter Smad3 protein levels (Fig. 6A). Therefore, we sought to determine whether EAF2 could affect the C-terminal phosphorylation of Smad3. TGF-β treatment could indeed enhance Smad3 phosphorylation, as revealed after detecting using anti-Smad3 (Ser(P)-423/425) antibody (Fig. 6C). Moreover, overexpression of EAF2 also did not affect Smad3 stability (Fig. 6B).

Activation of Smad3 involves C-terminal phosphorylation and oligomerization with Smad4 followed by subsequent nuclear translocation (10). Therefore, we sought to determine whether EAF2 could affect the C-terminal phosphorylation of Smad3. TGF-β treatment could indeed enhance Smad3 phosphorylation, as revealed after detecting using anti-Smad3 (Ser(P)-423/425) antibody (Fig. 6C). However, overexpression of EAF2 had no effect on endogenous Smad3 phosphorylation in the presence or absence of TGF-β (Fig. 6C). Moreover, knockdown of EAF2 also did not affect Smad3 phosphorylation (Fig. 6D). Therefore, EAF2 neither affects Smad3 protein stability nor affects Smad3 phosphorylation.

both the presence and absence of TGF-β (2 ng/ml) (Fig. 3C). Together, these results suggest that EAF2 specifically interacts with Smad3 in the both presence and absence of TGF-β.

Next, we mapped the domains of EAF2 that are responsible for interacting with Smad3 using deletion mutants (Fig. 4A1). As shown in Fig. 4, A2 and A3, the C terminus of EAF2, which consists of amino acids 162–260 comprising exons V-VI, was crucial for interaction with Smad3. Subsequently, we employed GST pulldown assays to map the domains of Smad3 that interact with EAF2. As shown in Fig. 4B, the MH2 domain (amino acids 200–426) interacted with EAF2 directly (10). Taken together, these results suggest that EAF2 directly and specifically interacts with Smad3.

EAF2 Inhibits Smad3 Transcriptional Activity—Because EAF2 associates with Smad3, but not with Smad1, Smad2, and Smad4 in vivo. FLAG-tagged Smad1, Smad2, Smad3, and Smad4 plasmid constructs were co-transfected with Myc empty vector or Myc-tagged EAF2 plasmid constructs into HEK293T cells. Anti-Myc antibody conjugated agarose beads were used for immunoprecipitation (IP) assays. TGF-β (2 ng/ml) was added to the medium. WB, Western blot. TCL, total cell lysate. B, EAF2-associated with Smad3 was further confirmed. HA empty vector or HA-tagged Smad1, Smad2, or Smad3 vector were co-transfected with Myc-tagged EAF2 into HEK293T cells. Bone morphogenetic protein 2 (BMP-2; 10 ng/ml) was added to HA-Smad1 and Myc-EAF2 co-transfection. TGF-β (2 ng/ml) was added to HA-Smad2/Smad3 and Myc-EAF2 co-transfection. C, EAF2 does not associate with Smad7. HA-tagged Smad3, Smad7, and HA empty vector were co-transfected with Myc-tagged EAF2 into HEK293T cells. TGF-β (2 ng/ml) was added to the medium. D, coimmunoprecipitation between endogenous EAF2 and endogenous Smad3 in HaCaT cells in the presence/absence of TGF-β (2 ng/ml). Anti-Smad3 antibody was used for immunoprecipitation and rabbit IgG was used as a control.

FIGURE 3. EAF2 specifically interacts with Smad3. A, EAF2 associates with Smad3, but not with Smad1, Smad2, and Smad4 in vivo. FLAG-tagged Smad1, Smad2, Smad3, and Smad4 plasmid constructs were co-transfected with Myc empty vector or Myc-tagged EAF2 plasmid constructs into HEK293T cells. Anti-Myc antibody conjugated agarose beads were used for immunoprecipitation (IP) assays. TGF-β (2 ng/ml) was added to the medium. WB, Western blot. TCL, total cell lysate. B, EAF2-associated with Smad3 was further confirmed. HA empty vector or HA-tagged Smad1, Smad2, or Smad3 vector were co-transfected with Myc-tagged EAF2 into HEK293T cells. Bone morphogenetic protein 2 (BMP-2; 10 ng/ml) was added to HA-Smad1 and Myc-EAF2 co-transfection. TGF-β (2 ng/ml) was added to HA-Smad2/Smad3 and Myc-EAF2 co-transfection. C, EAF2 does not associate with Smad7. HA-tagged Smad3, Smad7, and HA empty vector were co-transfected with Myc-tagged EAF2 into HEK293T cells. TGF-β (2 ng/ml) was added to the medium. D, coimmunoprecipitation between endogenous EAF2 and endogenous Smad3 in HaCaT cells in the presence/absence of TGF-β (2 ng/ml). Anti-Smad3 antibody was used for immunoprecipitation and rabbit IgG was used as a control.
Regulation of Smad3 nuclear translocation has been reported to be one of the mechanisms for modulation of Smad3 activity (19–26). To determine whether EAF2 can affect Smad3 nuclear translocation, we fractionated cells into nuclear and cytoplasmic preparations before preparing extracts. Overexpression of EAF2 could prevent Smad3 nuclear translocation in the presence of TGF-β/ß2 but not in the absence of TGF-β/ß2 (Fig. 6E). However, overexpression of EAF2 had no obvious effect on Smad2 nuclear translocation (Fig. 6E), consistent with that EAF2 only interacts with Smad3 revealed above.

Furthermore, we examined the effect of EAF2 knockdown on the nuclear translocation of Smad3. Even though the effect of EAF2 knockdown on the nuclear translocation of Smad3 was not as dramatic as that of EAF2 overexpression, knockdown of EAF2 could indeed enhance Smad3 nuclear translocation (Fig. 6F; 1.0 versus 1.28). But knockdown of EAF2 had no obvious effect on Smad2 nuclear translocation (Fig. 6F), consistent with that EAF2 only interacts with Smad3 revealed above.

To determine the correlation of EAF2 expression and Smad3 nuclear translocation physiologically, we examined the expressions of both EAF2 and Smad3 in human prostate specimens (n = 6) by immunohistochemical staining in parallel. Among these specimens, when EAF2 was highly expressed in the nuclei of prostate epithelium, Smad3 expression was relatively lower in the nuclei of the same region (n = 4) (Fig. 6G). But when EAF2 was expressed relatively lower in the prostate epithelium, Smad3 expression was relatively higher in the same region (n = 3) (Fig. 6H). Taken together, these observations suggest that EAF2 might attenuate TGF-β signaling by preventing Smad3 nuclear translocation.

**EAF2 Disrupts Smad3-Smad4 Complex Formation and Prevents p300 Binding to Smad3**—It has been shown that the oligomerization of Smad3 with Smad4 is required for Smad3 to exhibit its transcriptional activity (10, 29–31). To further elucidate the mechanism of EAF2 inhibition of Smad3 activity, we examined whether EAF2 binding can affect Smad3-Smad4 complex formation. Initially, we examined the effect of EAF2 on the enhancement role of Smad4 on Smad3 transcriptional activity. As shown in Fig. 7, A1 and A2, co-transfection of plasmid expressing Smad4 could indeed enhance Smad3 transcriptional activity, as revealed by promoter assays in Mv1Lu cells. Overexpression of EAF2 dramatically reduced Smad4 binding to Smad3 in the presence of TGF-β (Fig. 7B1). In contrast, knockdown of EAF2 by EAF2-shRNA-2 in HaCaT cells enhanced Smad4 binding to Smad3 in the presence of TGF-β as revealed by endogenous coimmunoprecipitation assays (Fig. 7B2). As previously reported, the transcriptional adaptor CBP/p300 is a coactivator for Smad3 in TGF-β induced transcrip-
To gain more insight into the mechanism behind the inhibitory role of EAF2 on Smad3 transcriptional activity, we examined whether EAF2 could affect the binding of p300 to Smad3. To monitor Smad3 transcriptional activity directly, we cloned Smad3 into the pM vector (Clontech, Mountain View, CA), which could express a fusion protein containing a GAL4 DNA binding domain. Overexpression of EAF2 could indeed inhibit Smad3 transcriptional activity induced by TGF-β (Fig. 8, B1 and B2). Further coimmunoprecipitation assays showed that overexpression of EAF2 diminished p300 binding to Smad3 in the presence of TGF-β. In contrast, knockdown of EAF2 by EAF2-shRNA-2 in HaCaT cells enhanced p300 binding to Smad3 in the presence of TGF-β, as revealed by endogenous coimmunoprecipitation assays (Fig. 8B2). Taken
together, these results suggest that EAF2 binding disrupts Smad3-Smad4 complex formation and impairs p300 recruitment to Smad3.

**EAF2 Attenuates TGF-β-induced Cell-cycle Arrest and Cell Migration**—One of the earliest identified roles of TGF-β signaling is its ability to arrest the cell cycle in early G1 phase, resulting in growth inhibition of various cell types (35). In addition, it is evident that TGF-β could promote cell migration and invasion (36–38). To demonstrate the biological consequences of EAF2 expression via inhibition of TGF-β signaling, we performed cell cycle analyses and cell growth assays as well as cell migration assays. Without TGF-β treatment, overexpression of
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EAF2 in HaCaT cells had no obvious effect on cell cycle (Fig. 9A). As expected, with TGF-β treatment, more cells were arrested in G1 phase (Fig. 9A). However, overexpression of EAF2 dramatically reduced the number of cells arrested in G1 phase dramatically (Fig. 9A). Consistently, the growth rate of EAF2 wild type MEF cells (EAF2+/+) was higher than that of EAF2-null MEF cells (EAF2−/−) in the presence of TGF-β (2 ng/ml) as revealed by cell growth assays (Fig. 9B).

Subsequently, we used EAF2-null MEF cells to perform cell migration assays. Without TGF-β treatment, no significant difference was observed in migration between EAF2 wild type MEF cells and EAF2-null MEF cells (Fig. 9C). The TGF-β treatment could indeed induce greater cell migration (Fig. 9C1). However, after TGF-β treatment, the number of EAF2-null cells that had migrated, was significantly larger than that observed for EAF2 wild type MEF cells (Fig. 9C2). These data suggest that EAF2 inhibits both TGF-β-induced cell cycle arrest at early G1 phase and TGF-β-induced cell migration.

Discussion

EAF2 Is a Novel Repressor of TGF-β Signaling—Given that Smad proteins are key mediators of TGF-β signaling, identification of their binding partners has attracted much attention (10). Using various approaches, many binding partners of Smad proteins have been characterized, serving either as co-activators or as co-repressors (12, 29, 30, 33, 34, 36, 39–43). Most of them may associate with more than one Smad protein (12, 30, 33, 43). In this study we found that EAF2 only interacts with...
Smad3 to suppress Smad3 transcriptional activity, resulting in inhibition of TGF-β signaling. Thus, EAF2 may represent a specific repressor of TGF-β signaling solely through its effect on Smad3 activity.

Smad protein binding partners affect TGF-β signaling through diverse mechanisms (10). Because the C-terminal phosphorylation of R-Smads mediated by type I TGF-β receptors is a relatively early, critical step in the activation of TGF-β signaling, R-Smad binding partners involved in TGF-β-induced R-Smad phosphorylation are often examined first (10). However, EAF2 binding does not affect Smad3 phosphorylation. Therefore, the inhibitory role of EAF2 on TGF-β signaling does not result from its effect on the level of TGF-β-induced phosphorylation of Smad3. Notably, several Smad3-specific associated proteins have been identified, which also affects TGF-β signaling through a Smad3 phosphorylation-independent mechanism, such as AKT (39, 40), SARS-associated coronavirus (SARS-CoV) nucleocapsid protein (13), and TRB3 (36). Thus, EAF2 may belong to this group of Smad3-associated factors, which affect TGF-β signaling through a mechanism independent of Smad3 phosphorylation.

The multiple functions of TGF-β signaling on tissue homeostasis and disease progression have been well demonstrated (8). Regulation of TGF-β signaling could attribute to affect tissue homeostasis and disease progression. To date, many regulators of TGF-β signaling have been shown to have important impacts on tissue homeostasis and disease progression (8, 9). Here, we identified EAF2 as a specific binding partner of Smad3 to suppress Smad3 activity, resulting in inhibition of TGF-β signaling. Thus, EAF2 may also act its biological function through specifically interacting with Smad3 to inhibit TGF-β signaling.

The EAF2 has been shown as an androgen up-regulated gene with tumor suppressive function (3–5). However, the underlying mechanisms remain largely unknown. Although the function of TGF-β signaling in tumorigenesis is paradoxical, which could work either as a tumor suppressor or as an oncogene, it is well documented that particularly at the late stage of tumorigenesis, TGF-β signaling mainly promotes cancer cell progression by modulating processes such as epithelial-mesenchymal transition, evasion of immunity, and metastasis (9, 10). In this study we found that EAF2 could diminish the effect of TGF-β-induced G1 cell cycle arrest, resulting in enhancement of cell proliferation in the presence of TGF-β. Obviously, this is a discrepancy for EAF2 tumor suppressive function. Of note, in the absence of TGF-β, overexpression of EAF2 could actually sup-
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FIGURE 9. EAF2 attenuates TGF-β-induced G1 cell cycle arrest and cell migration. A, in HaCaT cells, overexpression of EAF2 attenuated TGF-β-induced G1 cell cycle arrest. Without TGF-β treatment (2 ng/ml), the number of cells with EAF2 overexpression arrested in G1 phase was similar to that of the control (average 60% versus 62%, p = 0.0668; the second column compared with the first column). After TGF-β treatment (2 ng/ml), the number of cells with EAF2 overexpression arrested in G1 phase was significantly lower than that of the control without EAF2 overexpression (average 64% versus 71%, p = 0.0036; fourth column compared with the third column). TGF-β treatment caused more cells to be arrested in G1 phase (average 71% versus 71%, p = 0.0005). B, in the presence of TGF-β (2 ng/ml), the growth rate of EAF2 wild type MEF cells (EAF2+/+) was higher than that of EAF2-null MEF cells (EAF2−/−). C1, TGF-β-induced cell migration was enhanced significantly in EAF2-null MEF cells (EAF2−/−) as compared with that of EAF2 wild type MEF cells (EAF2+/+). Representative pictures for cell migration analysis. C2, quantitative analysis for cell migration. After TGF-β treatment (2 ng/ml), more EAF2-null MEF cells (EAF2−/−) were migrated as compared with EAF2 wild type MEF cells (EAF2+/+) (p < 0.0001). Data are presented as the mean ± S.E. of three independent experiments performed in triplicate.

press cell growth (3). Therefore, it appeared that the microenvironment (with or without TGF-β) could completely reverse the effect of EAF2 on cell proliferation. Further investigation of this phenomenon might shed new light on the function of EAF2 in tumor suppression.

In addition, in this study we also observed that EAF2 could indeed inhibit TGF-β-induced cell migration, which is consistent with the tumor suppressive function of EAF2. Therefore, partially at the late stage of tumorigenesis, EAF2 might still act its tumor suppressive function through inhibiting TGF-β signaling, at least in part.

EAF2 Inhibits Smad3 Transactivity via Reduction of Smad3 Nuclear Translocation, Disruption of Smad3-Smad4 Complex Formation, and Prevention of p300 Association—Smad3 nuclear translocation has been reported to be one of the crucial mechanisms for modulation of TGF-β signaling (19–26). Even though a major nuclear localization signal has been identified in the N terminus of Smad3 (19), a surface hydrophobic corridor within the MH2 domain of Smad3 was found to be necessary for association with CAN/Nup214 and nuclear import, which also participates in Smad3 nuclear translocation. In this study we revealed that EAF2 interacts with the MH2 domain of Smad3. Thus, EAF2 binding might interfere with Smad3 association with CAN/Nup214 to prevent subsequent nuclear import, resulting in cytoplasmic retention of Smad3.

The association of Smad3 with Smad4 has been proposed to be essential for the activation of TGF-β signaling (8–10). Thus, any factors that affect the association between R-Smads and Smad4 exhibit high impact on TGF-β signaling. In this study we observed that EAF2 binding competes with Smad4 to bind to Smad3 and inhibits Smad3 transcriptional activity. These data may provide another explanation for the mechanism whereby EAF2 inhibits TGF-β signaling. In fact, a similar explanation has been proposed for the effects of orphan nuclear receptor and SARS-CoV N protein on Smad3-mediated TGF-β signaling (13, 43).

Like many other transcription factors, Smad3 protein interacts with the paralogous transcriptional co-activators p300 and CBP through its MH2 domain (32, 44–46). These two proteins, p300 and CBP, act as a crucial scaffold to bring together transcription factors and basal factors to form the transcriptional initiation complex. An increasing number of nuclear activators including the AP-1 complex, vitamin D receptor, TFE3, hZimp10, SARS-CoV, nucleocapsid, and TRB3 protein have been shown to interact specifically with Smad proteins and to increase their transcriptional activities by enhancing CBP/p300 binding (13, 30, 31, 33, 36, 47–49). In addition, a number of repressors including Evi-1, Ski, SnoN, TGF, SIP1, SNIP1, AKT, Erbin, and Zili have been shown to interact with, as well as inhibit, Smad protein transcriptional activities by interfering with the ability of CBP/p300 to interact with Smad proteins (12, 33, 39–41, 50–53). Here, we show that EAF2 binds to the MH2 domain of Smad3, a region implicated in CBP/p300 binding, and inhibits Smad3 transcriptional activity directly, as revealed
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using mammalian one-hybrid assays. Moreover, we found that EAF2 binding could indeed prevent p300-Smad3 complex formation. Therefore, another mechanism for EAF2 in the inhibition of TGF-β signaling appears to act through disruption of p300 binding to Smad3. Taken together, EAF2 might inhibit TGF-β signaling through multiple pathways. However, the way that EAF2 participates in these pathways is still unclear. Further elucidation of the molecular events underlying these pathways will provide insight into the function of EAF2 in mechanistic terms.

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