# Dual roles of myocardin-related transcription factors in epithelial–mesenchymal transition via *slug* induction and actin remodeling

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Epithelial–mesenchymal transition (EMT) is a critical process occurring during embryonic development and in fibrosis and tumor progression. Dissociation of cell–cell contacts and remodeling of the actin cytoskeleton are major events of the EMT. Here, we show that myocardin-related transcription factors (MRTFs; also known as MAL and MKL) are critical mediators of transforming growth factor β (TGF-β) 1–induced EMT. In all epithelial cell lines examined here, TGF-β1 triggers the nuclear translocation of MRTFs. Ectopic expression of constitutive-active MRTF-A induces EMT, whereas dominant-negative MRTF-A or knockdown of MRTF-A and -B prevents the TGF-β1–induced EMT. MRTFs form complexes with Smad3. Via Smad3, the MRTF–Smad3 complexes bind to a newly identified cis-element GCCG-like motif in the promoter region of *Canis familiaris* and the human *slug* gene, which activates *slug* transcription and thereby dissociation of cell–cell contacts. MRTFs also increase the expression levels of actin cytoskeletal proteins via serum response factor, thereby triggering reorganization of the actin cytoskeleton. Thus, MRTFs are important mediators of TGF-β1–induced EMT.

## Introduction

Epithelial–mesenchymal transition (EMT) is a critical process occurring during embryonic development and in fibrosis and tumor progression (Lee et al., 2006; Thiery and Seleeman, 2006). TGF-β is a major inducer of EMT, which triggers dissociation of cell–cell contacts and remodeling of the actin cytoskeleton, permitting adherent epithelial cells to scatter and migrate directionally through the extracellular matrix (Zavadil and Bottinger, 2005). Binding of TGF-β to its receptor leads to phosphorylation of its downstream targets Smad2 and 3, and then the phosphorylated Smad2 and 3 form complexes with cytoplasmic Smad4 (Xu, 2006). The Smad complexes translocate into the nucleus where they regulate transcription of target genes through binding to specific cis-elements within their promoter regions (Zawel et al., 1998; Kusanagi et al., 2000). Recent studies suggest that Smads play a critical role in TGF-β–induced EMT by regulating transcription of their target genes (Zavadil et al., 2004; Valcourt et al., 2005). TGF-β is also

reported to activate several signaling cascades, such as the extracellular signal–related mitogen-activated protein kinase (Zavadil et al., 2001), p38 mitogen-activated protein kinase (Yu et al., 2002), phosphatidylinositol 3 kinase (Lamouille and Derynck, 2007), and Rho pathways (Bhowmick et al., 2001), which also contribute to TGF-β–induced EMT, respectively.

Many kinds of transcription factors, such as zinc finger transcriptional factors Snail (Batlle et al., 2000; Cano et al., 2000), Slug (Savagner et al., 1997), and basic helix-loop-helix transcription factor Twist (Yang et al., 2004), have been shown as regulators of EMT. They directly repress the transcription of E-cadherin, leading to dissociation of cell–cell contacts. Although TGF-β up-regulates the expression of these EMT regulators in some epithelial cell lines, the molecular mechanism underling their expressions is not fully understood. In this connection, two transcriptional regulators, the hairy/enhancer of split-related transcriptional repressor (Hey1) and high mobility group A2 (HMGA2), have been identified as more upstream regulators of EMT (Zavadil et al., 2004; Thuault et al., 2006). TGF-β1 stimulation rapidly and transiently induces the expression of *hey1* and *hmga2* genes via activation of Smad2/3 signaling, resulting in up-regulation of *snail* and *slug* expressions. It is, however, unclear how Hey1 and HMGA2 are involved in the expression of these EMT regulators.

Myocardin-related transcription factor (MRTF) family members MRTF-A and -B (also known as MAL and MKL1/2) have been reported to be coactivators of serum response factor (SRF)–dependent transcription (Wang et al., 2002; Miralles et al., 2003). Myocardin is restricted expressed in smooth and cardiac muscles and regulates the differentiation of these muscle types via transactivation of the appropriate differentiation marker genes (Wang et al., 2001). In contrast, MRTFs are broadly distributed in tissues and cells (Wang et al., 2002). Although MRTFs are also involved in muscle differentiation (Selvaraj and Prywes, 2003; Li et al., 2005), their functions in nonmuscle cells are largely unclear, except for regulation of mammary myoepithelial differentiation (Li et al., 2006; Sun et al., 2006). The activity of MRTFs is regulated via their nuclear translocation, which is triggered by activation of the Rho signaling pathway (Miralles et al., 2003), and TGF-β also affects the subcellular localization of MRTF-A (Fan et al., 2007; Hinson et al., 2007). Here, we investigated the involvement of MRTFs in TGF-β1–induced EMT and demonstrated that MRTFs induce the *slug* expression in response to TGF-β stimulation coupling with the Smad pathway. Additionally, MRTFs also regulate reorganization of the actin cytoskeleton mediated through transcriptional activation of actin cytoskeletal genes. Thus, MRTFs are critical mediators for TGF-β1–induced EMT with their dual functions.

## Results

### Rho-dependent activation of MRTFs by TGF-β1 induces EMT in MDCK cells

We first examined the location of MRTFs in the *Canis familiaris* kidney epithelial (MDCK) cells, and found both MRTF-A and -B mostly in the cytosol. In response to TGF-β1 stimulation, MRTF-A, but not -B, translocated into the nucleus after 24 h of the stimulation (Fig. 1 A). The nuclear translocation of MRTFs is reportedly regulated by Rho activity (Miralles et al., 2003). In MDCK cells, the Rho inhibitor exoenzyme C3 completely suppressed the nuclear translocation of MRTF-A (Fig. 1 A). We also monitored endogenous MRTF activity with an SRF-dependent luciferase reporter assay, using a construct containing the SRF-binding cis-elements (3xCArG). TGF-β1 enhanced the reporter activity after 12 to 24 h of stimulation, and Rho kinase inhibitor Y27632 or Rho inhibitor C3 suppressed this activation (Fig. 1 B). To clarify the MRTF dependency of this promoter activation, MDCK cells were transfected with dominant-negative (DN) MRTF-A lacking its N-terminal RPEL motifs and C-terminal transactivation domain. The DN–MRTF-A protein forms nonproductive heterodimers with endogenous MRTF-A and -B, and therefore inhibits the function of endogenous MRTFs (Cen et al., 2003; Selvaraj and Prywes, 2003). DN–MRTF-A suppressed the TGF-β1–induced activation of the 3xCArG-Luc construct (Fig. 1 B), suggesting that TGF-β1 enhances the MRTF-A's function by promoting its Rho-mediated nuclear translocation.

To analyze MRTFs' function in EMT, we isolated stable MDCK cell lines expressing constitutive-active (CA) MRTF-A and DN–MRTF-A, respectively. The CA–MRTF-A protein, which lacks its N-terminal RPEL motifs, localizes in the nucleus without TGF-β1 stimulation. The parental MDCK cells grew as mono-

layers maintained by cell–cell junctions. CA–MRTF-A clones, however, showed a mesenchymal morphology without TGF-β1 stimulation. That is, they grew as scattered, individual cells that formed stress fibers (Fig. 1, C and D; and Fig. S1, A and B, available at http://www.jcb.org.cgi/content/full/jcb.200708174/DC1). In contrast, DN–MRTF-A clones did not respond to TGF-β1 by entering EMT (Figs. 1 C and S1 C). To confirm the effect of the loss of MRTFs' function, we designed two siRNAs (siRNA1 and siRNA2) for each MRTF whose targeting sequences are specific to MRTF-A and -B. Consistent with our DN experiments, knockdown of MRTF-A and -B by the siRNAs suppressed the TGF-β1–induced EMT (Fig. 1 E).

In MDCK cells, treatment with TGF-β1 led to decrease in expression levels of epithelial markers E-cadherin and β-catenin and increase in expression of mesenchymal markers N-cadherin and vimentin (Fig. 1 F). In contrast, almost no such changes were seen in DN–MRTF-A clones in response to TGF-β1, and CA–MRTF-A clones expressed mesenchymal markers at high levels instead of epithelial markers, even in the absence of TGF-β1 (Fig. 1 F). Depletion of MRTF-A and/or -B by siRNAs also suppressed the TGF-β1–induced expression changes in epithelial and mesenchymal markers (Fig. 1 G). Double depletion of MRTFs yielded a more severe phenotype than depletion of either MRTF alone, suggesting that MRTF-A and -B are both important in EMT. As shown in Fig. 1 A, however, MRTF-B seemed not to translocate into the nucleus upon TGF-β1 stimulation. In various cell types, MRTFs continuously shuttle between the cytosol and the nucleus, and therefore they are mostly observed in the cytosol under steady-state conditions (Zaromytidou et al., 2006; Fan et al., 2007; Vartiainen et al., 2007). To clarify the translocation of MRTFs induced by TGF-β1, MDCK cells were treated with the nuclear export inhibitor leptomycin B (LMB). Although both MRTFs remained in the cytosol in the absence of TGF-β1, they translocated to the nucleus when TGF-β1 was added along with LMB (Fig. 2). Collectively, these data indicate that TGF-β1 enhances the Rho-dependent nuclear translocation of both MRTF-A and -B, resulting in induction of EMT in MDCK cells.

### MRTFs mediate TGF-β1–induced expression of the *slug* gene

Several transcriptional regulators, including Snail, Slug, and Twist, have been reported to induce EMT through the transcriptional repression of E-cadherin (Savagner et al., 1997; Batlle et al., 2000; Cano et al., 2000; Yang et al., 2004). In MDCK cells, TGF-β1 markedly induced the expression of *slug*, but not of *snail* or *twist*, within 24 h (Fig. 3 A). Meanwhile, Peinado et al. (2003) previously reported TGF-β1–induced *snail* expression in MDCK II cells. We confirmed that TGF-β1 weakly and transiently induced *snail* expression in MDCK II cells, but not in MDCK cells, whereas *slug* expression was highly and continually induced for 3 d in both cell lines (Fig. 3 B). In our CA–MRTF-A clones, *slug* expression was strikingly higher than that in the parental cells, and the TGF-β1–induced up-regulation of *slug* expression was suppressed in DN–MRTF-A clones and MRTF-A/B–depleted cells (Fig. 3, C–E). Bolos et al. (2003) reported that the ectopic expression of *slug* was sufficient to
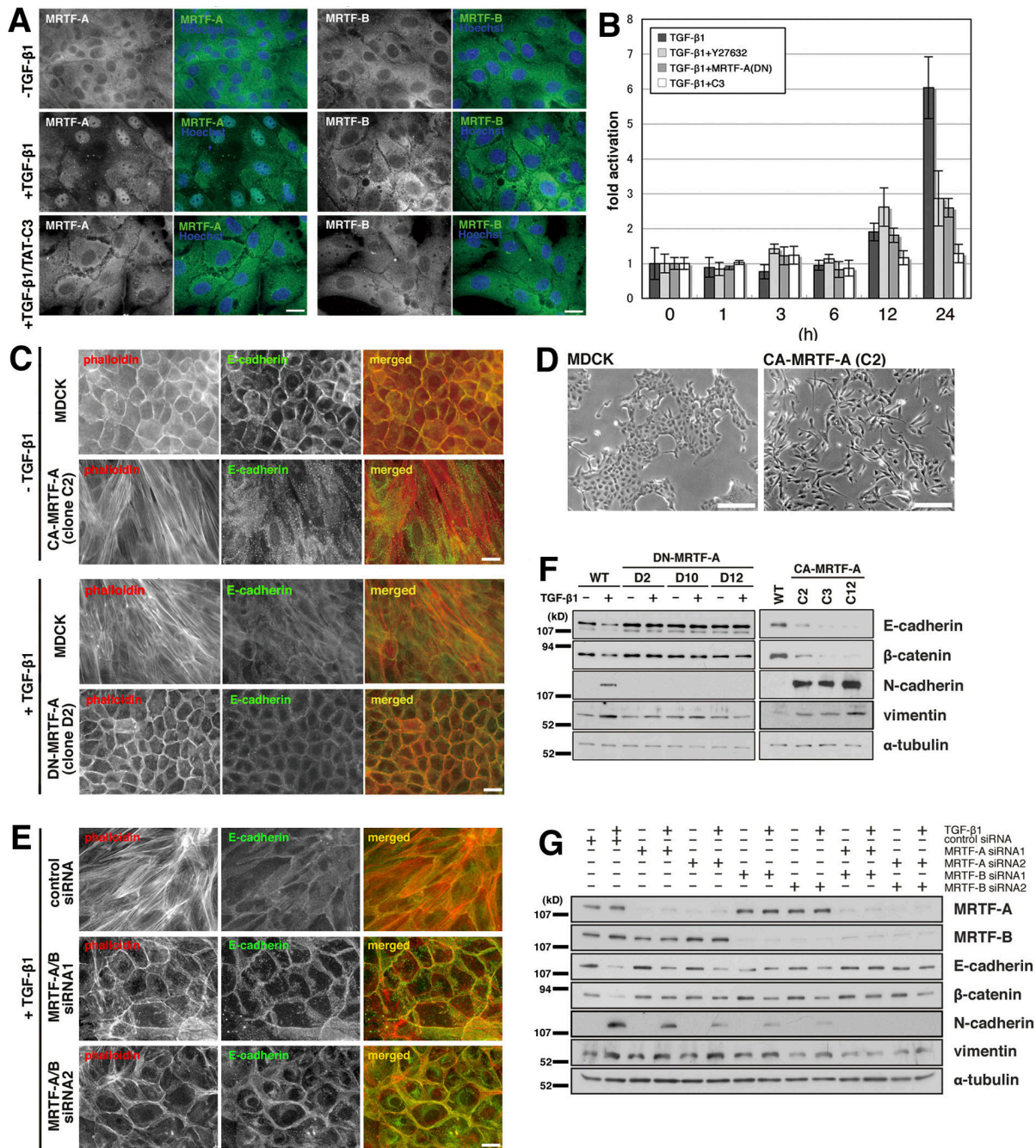
Figure 1. **Involvement of MRTFs in TGF-β1–induced EMT.** (A) MDCK cells were cultured with or without TGF-β1 for 24 h. In experiments using Rho inhibitor TAT-C3, 50 μg/ml TAT-C3 was added to the culture medium before TGF-β1 stimulation. The cells were fixed and stained with anti–MRTF-A or -B antibodies (green). The nuclei were stained with Hoechst 33342 (blue). Bars, 20 μm. (B) MDCK cells were transfected with 3xCArG-Luc, with or without pcDNA3.1–DN–MRTF-A or pcDNA3.1-C3. 18 h after the transfection, the cells were treated with TGF-β1 for the indicated number of hours and the luciferase activities were measured. In experiments using Rho kinase inhibitor Y27632, 20 μM Y27632 was added to the culture medium along with TGF-β1. Error bars represent SD from three independent experiments. (C) CA–MRTF-A cells (clone C2), DN–MRTF-A cells (clone D2), and parental MDCK cells (WT) were cultured with or without TGF-β1 for 24 h, and then stained with anti–E-cadherin antibody (green) and Alexa 568–conjugated phalloidin (red). Bars, 20 μm. (D) Phase-contrast images of MDCK and CA–MRTF-A cells (clone C2), which were cultured in DME-FCS without TGF-β1. Bars, 50 μm. (E) MDCK cells were transfected with siRNAs against MRTF-A (MRTF-A siRNA1 or 2) and -B (MRTF-B siRNA1 or 2). Scrambled siRNA was used as the control. After the transfection, the cells were cultured with or without TGF-β1 for 24 h, and then stained with anti–E-cadherin antibody (green) and Alexa 568–conjugated phalloidin (red). Bar, 20 μm. (F) DN–MRTF-A cells (clones D2, D10, and D12) and parental MDCK cells were cultured with or without TGF-β1 (left). CA–MRTF-A (clones C2, C3, and C12) and parental MDCK cells were cultured without TGF-β1 (right). The expression levels of epithelial and mesenchymal marker proteins were compared by immunoblotting. (G) MDCK cells were transfected with MRTF-A and -B siRNAs or control siRNA, and then cultured with or without TGF-β1. The expression levels of MRTF-A and -B and epithelial and mesenchymal marker proteins were compared by immunoblotting.

Figure 2. **Subcellular localization of MRTF-A and -B under the LMB-treated conditions.** MDCK cells were cultured with or without TGF-β1 for 24 h, and then treated with LMB for 1 h. The cells were fixed and stained with anti–MRTF-A and -B antibodies (green), Hoechst 33342 (blue), and Alexa 568–conjugated phalloidin (red). Bar, 20 μm.
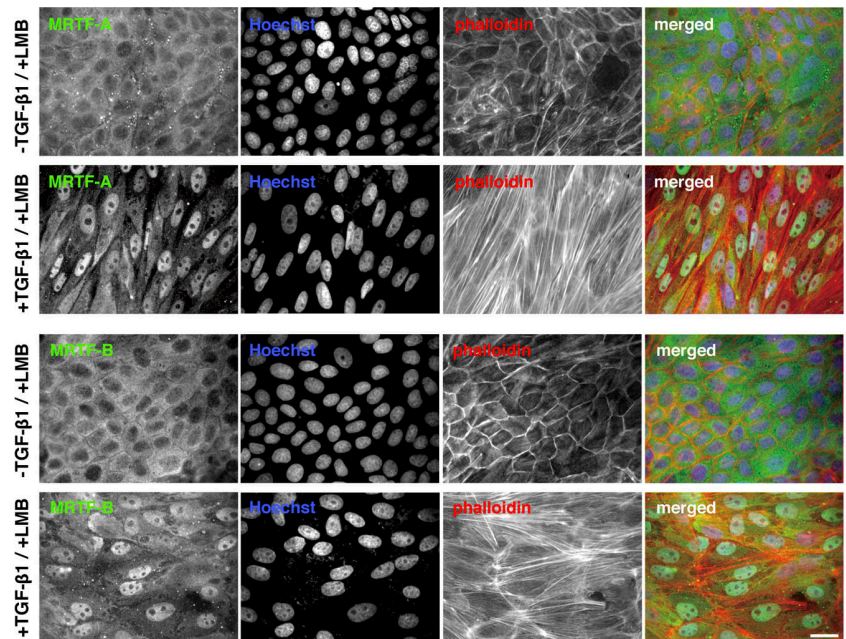


down-regulate epithelial marker expression and up-regulate mesenchymal markers in MDCK cells. Collectively, these results imply that MRTFs activated by TGF-β1 predominantly induce *slug* expression, leading to EMT in MDCK cells. Although TGF-β1 did not increase *twist* expression in MDCK cells, CA–MRTF-A clones exhibited high *twist* expression (Fig. 3 C). In DN–MRTF-A clones and MRTF-A/B–depleted cells, the basal levels of *twist* expression were lower than those in wild-type cells (Fig. 3, D and E). Thus, *twist* expression is TGF-β1 independent but MRTF dependent.

### MRTFs and Smad3 cooperatively regulate the *C. familiaris slug* promoter activity via a newly identified cis-element

To determine the regulatory mechanism underlying the transcription of *slug* gene by MRTFs, we analyzed the promoter region of the *C. familiaris slug* gene. Within the ∼2.5-kb fragment upstream of the first exon, we found several candidate cis-elements for SRF, Smad, and SP1 (Fig. 4 A and Fig. S2, available at http://www.jcb.org/cgi/content/full/jcb.200708174/DC1). To identify the MRTF-response elements, a series of 5′ promoter deletion mutants of the *slug* gene (F1–F8) were created. The F1–F6 reporter constructs were equally activated by MRTF-A, but this response was completely eliminated in F7 and F8, indicating that the MRTF-response elements lie between −218 and −293. This region has two types of putative binding sequences: two for SP1 (SP1near and SP1far) and one for Smad (GCCG box–like motif; Fig. 4 B). The mutational analysis revealed that the GCCG-like and SP1near motifs were important for the basal-level and/or MRTF-dependent activity, and therefore the doubly mutated construct (F6-mutGCCG/SP1near) lost its responsiveness to MRTF-A, and showed the same activity as F7 (Fig. 4, C and D). The F1 reporter construct was also activated by TGF-β1 stimulation, and depletion of MRTF-A/B by their siRNAs led to decrease in the TGF-β1–induced activation (Fig. 4 E).

Further, the GCCG-like and SP1near motifs were necessary for TGF-β1–induced activation of the *C. familiaris slug* promoter (Fig. 4 F). Thus, these results indicate that TGF-β1–MRTF signaling activates the *slug* promoter-reporter construct via the GCCG-like/SP1near motifs.

To determine whether the GCCG-like and SP1near motifs are sufficient for the TGF-β1 and MRTF responses, nine tandem copies of the sequence between −219 and −244 (core region) were inserted upstream of a reporter gene (9xcore region–Luc; Fig. 5 A). This reporter construct was significantly activated by TGF-β1, dependent on MRTFs' expression (Fig. 5 B). The GCCG motif has been identified as the *Drosophila melanogaster* Smad protein (Mad/Medea)–binding cis-element (Kusanagi et al., 2000). The reporter assay demonstrated that Smad1, 2, or 3 were not activators of 9xcore region–Luc, whereas MRTF-A markedly activated this reporter construct (Fig. 5, C and D). The coexpression of Smads with MRTF-A, however, slightly increased the activity of this construct in MDCK cells compared with MRTF-A alone (Fig. 5 C). The synergistic effect between MRTF-A and Smad3 on the activation of 9xcore region–Luc was definitely observed in HepG2 cells (Fig. 5 D). HepG2 cells showed robust activations of 9xcore region–Luc compared with MDCK cells, which may be because of differences in their cellular contexts, such as endogenous Smad3 and MRTF-A activities and transfection efficacies. Furthermore, depletion of endogenous Smad3 by its siRNAs reduced the responsiveness of 9xcore region–Luc to exogenous expression of MRTF-A (Fig. 5, E and F). Thus, these results suggest that MRTFs and Smad3 coordinately regulate the transcription of *slug* gene via the core region.

### MRTFs bind to the C terminus of Smad3 via their basic domains

Two research groups recently reported that myocardin binds to Smad1 and 3 and activates cardiac and smooth muscle– specific transcription (Callis et al., 2005; Qiu et al., 2005). In MDCK cells,
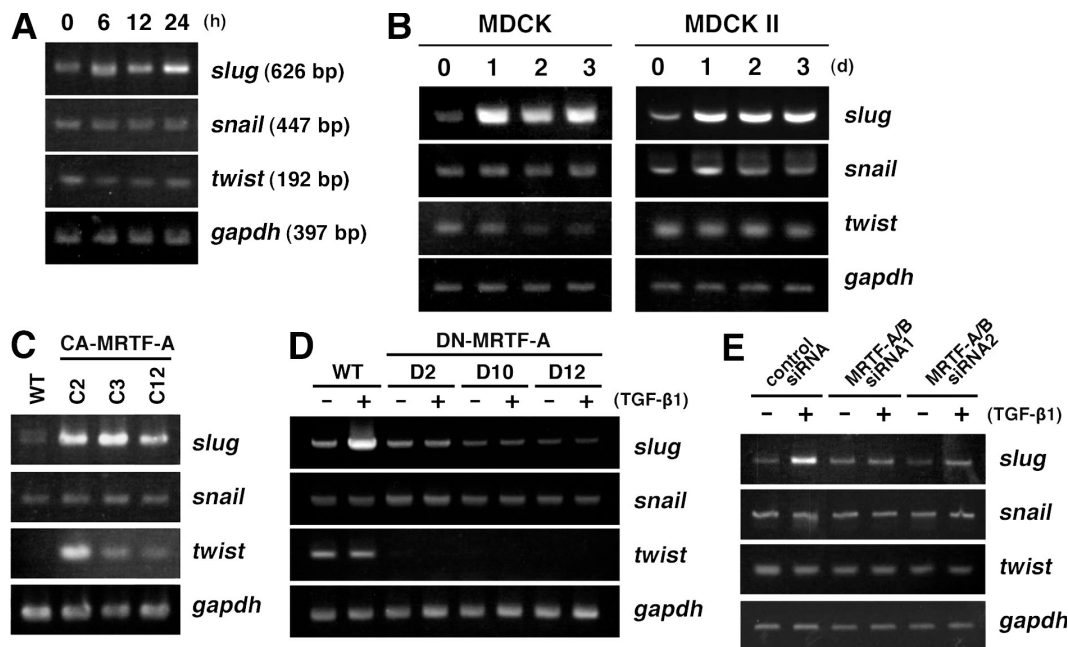
Figure 3. **Expression profiles of the *slug*, *snail*, and *twist* genes.** (A) MDCK cells were cultured with TGF-β1 for the indicated number of hours, and the expression time of EMT-inducible genes (*slug*, *snail*, and *twist*) was analyzed by RT-PCR. (B) MDCK or MDCK II cells were cultured with TGF-β1 for the indicated number of days, and the expression time of *slug*, *snail*, and *twist* genes was analyzed by RT-PCR. (C) The expression levels of *slug*, *snail*, and *twist* genes in CA–MRTF-A (clones C2, C3, and C12) and their parental MDCK cells were compared by RT-PCR. (D) DN–MRTF-A (clones D2, D10, and D12) and their parental MDCK cells were cultured with or without TGF-β1 for 24 h. The expression levels of *slug*, *snail*, and *twist* genes were compared by RT-PCR. (E) MDCK cells were transfected with MRTF-A or -B siRNAs or control siRNA, and the cells were cultured with or without TGF-β1 for 24 h. The expression levels of *slug*, *snail*, and *twist* genes were compared by RT-PCR.

endogenous MRTF-A and -B interacted with Smad3, and TGF-β1 treatment significantly increased their interactions (Fig. 6 A), suggesting that TGF-β1 not only induces nuclear translocation of MRTFs but also enhances their interactions. To determine the interacting domains of MRTFs and Smad3, deletion series of MRTF and Smad3 proteins were synthesized in vitro and their interactions were analyzed by coimmunoprecipitation. Smad3-C bound more tightly to MRTF-A–full than did Smad3-full, whereas the interaction between Smad3-NL and MRTF-A–full was very weak, indicating that the C-terminal domain of Smad3, including the MH2 domain, is largely responsible for the interaction with MRTF-A (Fig. 6, B and C). In contrast, Smad3-C bound to MRTF-A–full, MRTF-A N-term, and MRTF-A N-term ΔQ, but not to MRTF-A cent, MRTF-A C-term, or MRTF-A N-term ΔBQ (Fig. 6, D and E), indicating that the basic domain of MRTF-A is necessary for Smad3 binding. We confirmed that like MRTF-A, MRTF-B also bound to Smad3, and both MRTF-A ΔB and -B ΔB lost Smad3-binding activities (Fig. 6 F). These results indicate that the basic domain of MRTFs and the MH2 domain of Smad3 interact directly with each other.

**MRTF–Smad3 complex directly binds to the core region of *C. familiaris slug* promoter**
To determine whether the MRTF–Smad3 complexes bind to the DNA sequence within the *slug* core region, DNA–protein binding assay was performed using in vitro–translated HA-Smad3, FLAG–MRTF-A, and a biotinylated DNA probe containing the core region sequence. Kusanagi et al. (2000) reported that Smad1 and 4 directly bind to the GCCG motif (GCCGnCGC),

but Smad3 does not. Our present result, however, showed a significant interaction between Smad3 and the core region probe, which contained the GCCG-like motif (GCCGtCCC; Fig. 7 A). Expressed alone, MRTF-A did not bind to the DNA probe, although it bound significantly in the presence of Smad3 (Fig. 7 A). When a nonbiotinylated probe was added as a competitor, this interaction was markedly suppressed. Mutation of the GCCG-like motif in the competitor abrogated this interference, indicating that the MRTF–Smad3 complex binds to the core region of *slug* promoter via a Smad3–GCCG-like motif interaction. Furthermore, MRTF-A enhanced the affinity of Smad3 with the probe in a dose-dependent manner (Fig. 7 B). We also confirmed the interactions between MRTFs, Smads, and the core region sequence in vivo with chromatin immunoprecipitation (ChIP) assay and clearly showed that MRTF-A/B and Smad3, but not Smad1 or 2, bind to the core region of *slug* promoter in response to TGF-β1 stimulation (Fig. 7 C).

**MRTFs also regulate reorganization of the actin cytoskeleton in TGF-β1–induced EMT**
During EMT, TGF-β1 induces stress fiber formation instead of disappearance of cortical actin bundles. We demonstrated that TGF-β1–stimulated MDCK cells and CA–MRTF-A clones exhibit marked stress fiber formation with the loss of cortical actin bundles (Fig. 1 C and Fig. 8 A). The actin remodeling in TGF-β1–induced EMT appears to be regulated through the Rho pathway, which modulates reorganization of the actin cytoskeleton by actin polymerization and depolymerization (Bhowmick et al., 2001). Unexpectedly, the TGF-β1–induced
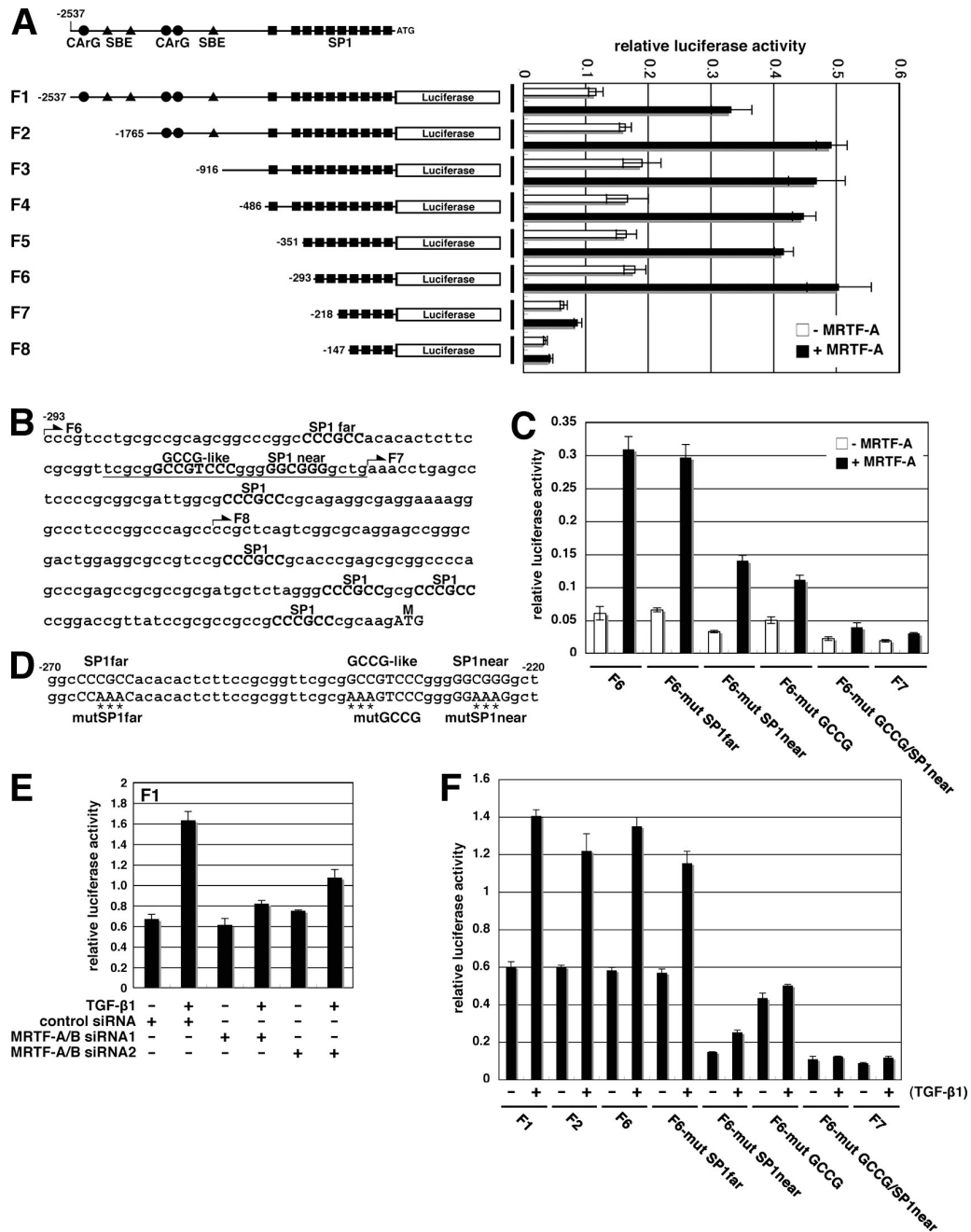
Figure 4. **Promoter analysis of the *C. familiaris slug* gene.** (A) Luciferase reporter assays using a series of 5' promoter deletion mutants of the *slug* gene (F1–F8). The ~2.5-kb fragment upstream of the first exon contains the putative CArG box–like sequences, SBE, and SP1-binding elements. MDCK cells were transfected with these reporter constructs with or without pcDNA3.1–CA–MRTF-A, and the luciferase activities were measured 24 h after the transfection. Error bars represent SD from three independent experiments. (B) Sequence for promoter construct F6, which bears seven SP1-binding elements and the GCCG-like motifs. The core region of the MRTF-A response elements is underlined. (C) Luciferase reporter assay using a series of mutated F6 constructs, in which sites in SP1far, SP1near, and/or the GCCG-like motifs were mutated as indicated in D. Error bars represent SD from three independent experiments. (E) MDCK cells were transfected with MRTF-A and -B siRNAs or control siRNA. After the transfection, the F1 reporter construct was introduced into the cells. The cells were cultured with or without TGF-β1 for 24 h, and then the luciferase activities were measured. Error bars represent SD from three independent experiments. (F) MDCK cells were transfected with the indicated reporter constructs. The cells were then cultured with or without TGF-β1 for 24 h and the luciferase activities were measured. Error bars represent SD from three independent experiments.

actin remodeling was completely suppressed by the protein synthesis inhibitor cycloheximide (Fig. 8 A), suggesting de novo protein synthesis is required. The expression of α–smooth muscle actin (α-SMA), a well-known mesenchymal marker, increased during EMT (Fig. 8 B). Moreover, we observed the up-regulation of caldesmon and tropomyosin (Fig. 8 B), which enhance stress fiber formation in various cell lines (Sobue and Sellers, 1991; Li et al., 2004). β-Actin protein slightly increased, but the expression levels of focal adhesion proteins vinculin and talin were unchanged (Fig. 8 B). Recently, we reported that
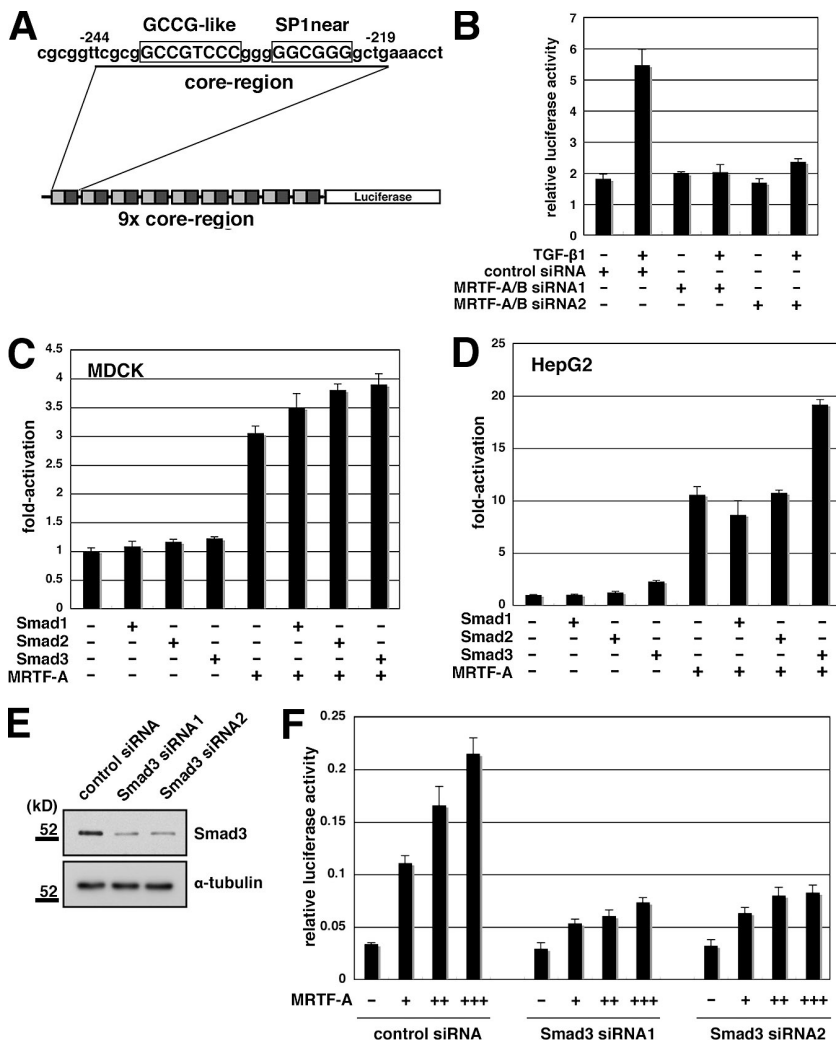
**A**
-244
cgcggttcgcgGCCGTCCCgggGGCGGGgctgaaacct -219
GCCG-like  SP1near
core-region

9x core-region | Luciferase

**B**
relative luciferase activity

| TGF-β1 | − | + | − | + | − | + |
| control siRNA | + | + | − | − | − | − |
| MRTF-A/B siRNA1 | − | − | + | + | − | − |
| MRTF-A/B siRNA2 | − | − | − | − | + | + |

**C** MDCK
fold-activation

| Smad1 | − | + | − | − | − | + | − | − |
| Smad2 | − | − | + | − | − | − | + | − |
| Smad3 | − | − | − | + | − | − | − | + |
| MRTF-A | − | − | − | − | + | + | + | + |

**D** HepG2
fold-activation

| Smad1 | − | + | − | − | − | + | − | − |
| Smad2 | − | − | + | − | − | − | + | − |
| Smad3 | − | − | − | + | − | − | − | + |
| MRTF-A | − | − | − | − | + | + | + | + |

**E**
control siRNA, Smad3 siRNA1, Smad3 siRNA2
(kD)
52 — Smad3
52 — α-tubulin

**F**
relative luciferase activity

| MRTF-A | − | + | ++ | +++ | − | + | ++ | +++ | − | + | ++ | +++ |

control siRNA    Smad3 siRNA1    Smad3 siRNA2

Figure 5. **Promoter analysis of the 9xcore region–Luc construct.** (A) Schematic representation of the 9xcore region–Luc construct. (B) MDCK cells were transfected with MRTF-A or -B siRNAs or control siRNA. After the transfection, the 9xcore region–Luc construct was introduced into the cells. The cells were cultured with or without TGF-β1 for 24 h, and then the luciferase activities were measured. Error bars represent SD from three independent experiments. (C and D) The 9xcore region–Luc construct was introduced into MDCK (C) or HepG2 (D) cells together with pcDNA3.1-Smad1(3E), pcDNA3.1-Smad2(2E), pcDNA3.1-Smad3(3E), and/or pcDNA3.1–CA–MRTF-A, and the luciferase activities were measured 24 h after the transfection. Error bars represent SD from three independent experiments. (E) HepG2 cells were transfected with two Smad3 siRNAs (Smad3 siRNA1 or 2) or control siRNA, respectively. 24 h after the transfection, the expression levels of Smad3 protein were analyzed by immunoblotting. (F) HepG2 cells were transfected with Smad3 siRNAs or control siRNA. After the transfection, the 9xcore region–Luc construct was introduced into the cells with various amounts of pcDNA3.1–CA–MRTF-A (0 μg [−], 0.3 μg [+], 0.6 μg [++], and 0.9 μg [+++]), and the luciferase activities were measured. Error bars represent SD from three independent experiments.

MRTF-A and -B regulate reorganization of the actin cytoskeleton in fibroblast NIH 3T3 cells through transcriptional regulation of actin cytoskeletal genes, including *caldesmon* and *tropomyosin 1* (Morita et al., 2007). In CA–MRTF-A clones, expression levels of the actin cytoskeletal proteins were markedly increased (Fig. 8 C). In contrast, the TGF-β1–induced up-regulation of these proteins was severely repressed in DN–MRTF-A clones (Fig. 8 C). The depletion of MRTF-A/B also repressed the TGF-β1 induction of cytoskeletal proteins (Fig. 8 D), suggesting that MRTFs activate the transcription of these genes in response to TGF-β1 stimulation. Importantly, the promoter regions of *caldesmon* (Yano et al., 1995), *tropomyosin* (Nakamura et al., 2001), α-*SMA* (Blank et al., 1992), and β-*actin* (Liu et al., 1991) all contain a CArG box or CArG box–like motif (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200708174/DC1) and their transcription is regulated by SRF. In our reporter assay, TGF-β1 enhanced the activities of these promoters in a CArG box–dependent manner (Fig. 8 E) and these activations were suppressed by DN–MRTF-A (Fig. 8 F). These results strongly suggest that once activated by TGF-β1, MRTFs also enhance the SRF/CArG-mediated transcription of actin cytoskeletal genes, which leads to remodeling of the actin cytoskeleton.

## MRTFs regulate TGF-β1–induced EMT in HK-2 and NMuMG cells

To confirm whether our current model, demonstrated in MDCK cells, is applicable to other cell types, we isolated CA–MRTF-A– and DN–MRTF-A–expressing stable cell lines of mouse mammary epithelial NMuMG cells and human renal proximal tubular epithelial HK-2 cells. Like MDCK cells, MRTFs expressed in NMuMG were translocated into the nucleus by TGF-β1 stimulation in the presence of LMB, whereas HK-2 cells showed the TGF-β1–induced nuclear translocation of MRTFs even without LMB (Fig. S4, available at http://www.jcb.org/cgi/content/full/jcb.200708174). In NMuMG and HK-2 clones expressing CA–MRTF-A, EMT events, namely actin remodeling associated with up-regulation of actin cytoskeletal proteins, dissociation of cell–cell adhesion, and expressional changes from epithelial to mesenchymal markers were observed without TGF-β1 stimulation (Fig. 9, A–E). In contrast, EMT events induced by TGF-β1 were suppressed in NMuMG and HK-2 clones expressing DN–MRTF-A (Fig. 9, A–E).

In NMuMG cells, TGF-β1 up-regulated the expression of *slug*, *snail*, and *twist*, and these up-regulations were maintained for at least 3 d after TGF-β1 stimulation (Fig. 9 F). Ectopic CA–MRTF-A markedly induced the expression of *slug*, *snail*, and *twist*,
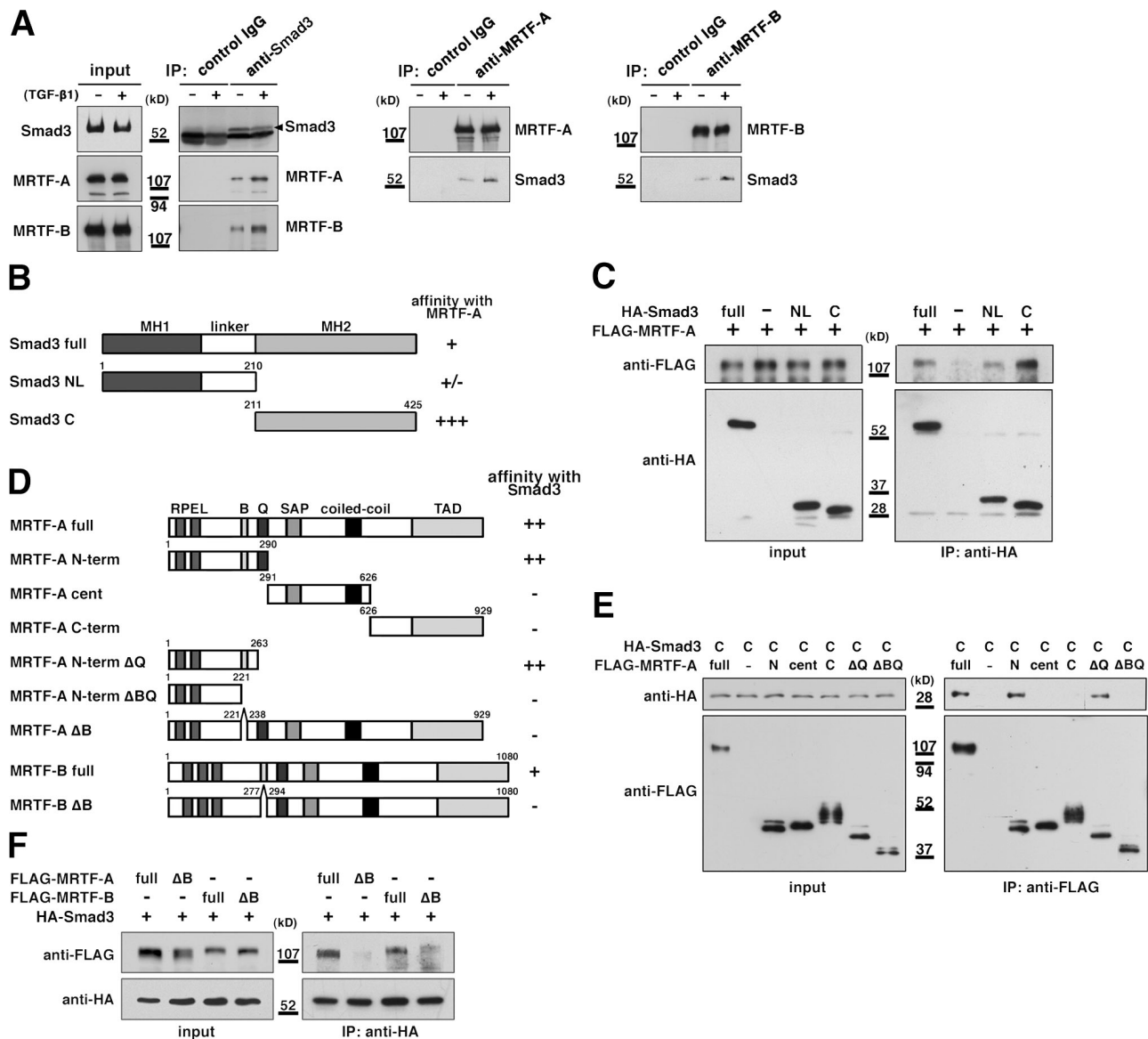
Figure 6. **Interactions between MRTF-A or -B and Smad3.** (A) Coimmunoprecipitation of the MRTFs and Smad3 expressed in MDCK cells. MDCK cells were cultured with or without TGF-β1 for 24 h. Coimmunoprecipitations were performed using nonimmune rabbit IgG (control IgG), anti-Smad3, or anti–MRTF-A or -B antibodies. The proteins in the precipitants were detected by immunoblotting with the indicated antibodies. (B) Schematic representation of the truncated mutants of Smad3. The MH1 domain, linker region, and MH2 domain are shown. The indicated numbers present amino acid positions. (C) HA-Smad3 full, HA-Smad NL, HA-Smad C, and full-length FLAG–MRTF-A proteins were synthesized in vitro, and their interactions were analyzed by coimmunoprecipitation using anti-HA antibody. (D) Schematic representation of the truncation series of MRTF-A and -B proteins. The RPEL motif, basic domain (B), Q-rich domain (Q), SAP domain, coiled-coil domain, and transactivation domain (TAD) are shown. (E) Truncation series of FLAG–MRTF-A proteins and HA-Smad3 C proteins were synthesized in vitro, and their interactions were analyzed by coimmunoprecipitation using anti-FLAG antibody. (F) FLAG–MRTF-A full, -B full, -A ΔB, and -B ΔB and HA-Smad3 full proteins were synthesized in vitro, and their interactions were analyzed by coimmunoprecipitation using anti-HA antibody.

whereas ectopic DN–MRTF-A suppressed the TGF-β1–induced expression of these genes (Fig. 9 G), indicating that TGF-β1–MRTF signaling regulates the induction of all these genes in NMuMG cells. In contrast, HK-2 cells exhibited TGF-β1 induction of *slug* expression but not of *twist* expression (Fig. 9, H and I). TGF-β1 also increased *snail* expression in HK-2 cells, but this induction was transient, just as observed in MDCK II cells (Fig. 9 H). In HK-2 cells, ectopic CA–MRTF-A markedly induced *slug* expression, and ectopic DN–MRTF-A suppressed the TGF-β1–induced *slug* expression (Fig. 9 I). Both CA–MRTF-A and DN–MRTF-A, however, did not affect *snail* and *twist* expressions

(Fig. 5, H and I), suggesting that MRTFs don't directly link to their expressions in HK-2 cells. Thus, *slug* expression was closely regulated by the TGF-β1–MRTF signaling in NMuMG and HK-2 cells, just as observed in MDCK cells, whereas *snail* and *twist* expressions were variously regulated in these cell lines.

## MRTF–Smad3 complex regulates the human *slug* promoter activity via GCCG-like motif

We further analyzed the molecular mechanism of human *slug* transcription by MRTFs and Smad3. Within the ∼1.1-kb fragment
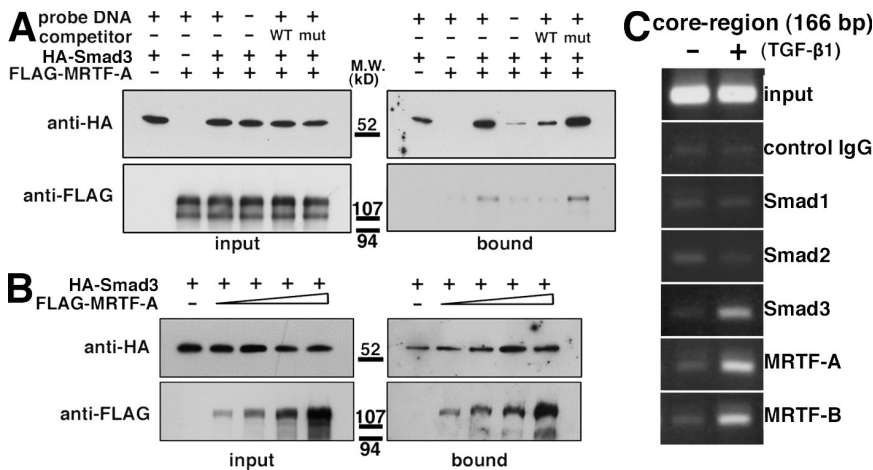
Figure 7. **Binding of MRTF/Smad3 to the core region sequence of *C. familiaris slug* promoter.** (A) DNA–protein binding assay was performed using a biotinylated core region DNA probe and in vitro–synthesized HA-Smad3 full and FLAG–MRTF-A full. The proteins that bound to the DNA probe were detected by immunoblotting with the indicated antibodies. (B) Synergistic effect of MRTF-A on the binding of Smad3 to the DNA probe containing the core region in a dose-dependent manner. (C) ChIP assay for MRTF-A and -B and Smad1, 2, and 3 binding to the endogenous core region sequence of the *slug* gene. MDCK cells were cultured with or without TGF-β1 for 24 h, and the ChIP assay was performed using nonimmune rabbit IgG (control IgG), anti–MRTF-A and -B, and anti-Smad1, 2, and 3 antibodies. PCR was performed to determine the *slug* promoter sequence surrounding the core region.

upstream of the first *slug* exon, there are several candidate cis-elements, including the GCCG-like motif (GCCGgCCC; Fig. 10, A and B; and Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200708174/DC1). Reporter assays using a series of human *slug* promoter constructs bearing point mutations showed that TGF-β1 and MRTF/Smad3 activated the human *slug* promoter mainly via the GCCG-like motif, similar to the *C. familiaris slug* promoter (Fig. 10, C and D). DNA–protein binding assay revealed that the MRTF–Smad3 complex bound to a DNA probe containing the GCCG-like motif of human *slug* gene, but not to a mutated one (Fig. 10 E). ChIP assay further showed the binding of MRTF/Smad3 to the GCCG-like motif of human *slug* promoter in a TGF-β1–dependent manner (Fig. 10 F). In the reporter assay, a Smad-binding element (SBE) appeared to be slightly involved in the activation of *slug* promoter construct by TGF-β1 and MRTF/Smad3 (Fig. 10, C and D). The results of DNA–protein binding and ChIP assays, however, indicate that the binding of MRTFs/Smad3 to the SBE was faint compared with that to the GCCG-like motif (Fig. 10, G–I). Thus, the contribution of the SBE to *slug* promoter activity is rather weak. In addition to the GCCG-like motif and SBE, two CArG box–like sequences also contribute to the responsibility of the *slug* promoter construct to exogenous MRTF (Fig. 10 D), raising a possibility that, in addition to the GCCG-like motif, the CArG box–like sequences may be synergistically involved in the TGF-β1–mediated induction of human *slug* gene.

## Discussion

The studies presented here clearly demonstrated that TGF-β1 triggers the nuclear translocation of MRTFs, which activates the two parallel pathways during EMT (Fig. 10 J). One pathway up-regulates the expression of EMT-regulating genes, such as *slug*, via MRTFs, Smad3, and GCCG-like motifs, leading to dissociation of cell–cell contacts. The other up-regulates the expression of actin cytoskeletal genes via MRTFs, SRF, and CArG box, resulting in remodeling of the actin cytoskeleton.

Recent studies demonstrate that MRTFs associate with monomeric G-actin through their RPEL motifs, which anchor MRTFs in the cytoplasm (Miralles et al., 2003; Posern et al., 2004).

Activated Rho reduces the cytoplasmic G-actin pool by enhancing actin polymerization, and then triggers the dissociation of MRTFs from G-actin, resulting in the nuclear translocation of MRTFs. TGF-β1 stimulation enhances the Rho activity in many kinds of epithelial cell lines (Bhowmick et al., 2001, 2003; Tian et al., 2003). We demonstrated that TGF-β1 induces the nuclear translocation of MRTF via activation of the Rho pathway. However, MRTF-A began to accumulate in the nucleus 12–24 h after TGF-β1 stimulation, and the CArG promoter construct was also activated by TGF-β1 with a similar time course (Fig. 1, A and B). These responses are rather slow for a direct regulation by activated Rho, raising the possibility that an additional factor is required for the nuclear accumulation of MRTFs. Vartiainen et al. (2007) recently demonstrated that the nuclear/cytosolic localization of MRTF-A and -B is determined by the balance between nuclear import and export. LMB treatment alone did not lead to accumulation of MRTF-A and -B in the nucleus. In TGF-β1–treated MDCK cells, nuclear import of MRTF-A would surpass its nuclear export, and thereby MRTF-A was accumulated in the nucleus (Fig. 1 A). In our and other research group's studies, MRTF-B seems to have a lower ability in nuclear import or a higher ability in nuclear export (Fan et al., 2007; Morita et al., 2007). In fact, nuclear accumulation of MRTF-B was observed in TGF-β1–treated MDCK cells only under the LMB-treated conditions (Fig. 2). These differences may be caused by different affinities of MRTF-A and -B with nuclear import or export machineries. TGF-β1 is also known to trigger the nuclear translocation of Smad complexes mediated through their phosphorylation (Xu, 2006). This raises a possibility that the Smad complexes may take MRTFs into the nucleus through their direct interactions after TGF-β1 stimulation. However, it is generally accepted that the nuclear translocation of Smad complexes is promptly occurring after TGF-β1 stimulation in a Rho-independent manner, suggesting that the nuclear translocation of Smad and MRTF are regulated by different mechanisms.

Recent studies have demonstrated that several transcriptional regulators, such as Slug, Snail, and Twist, are involved in EMT (Savagner et al., 1997; Batlle et al., 2000; Cano et al., 2000; Yang et al., 2004). Because it is difficult to obtain suitable Slug antibody for immunoblotting, we analyzed the expression
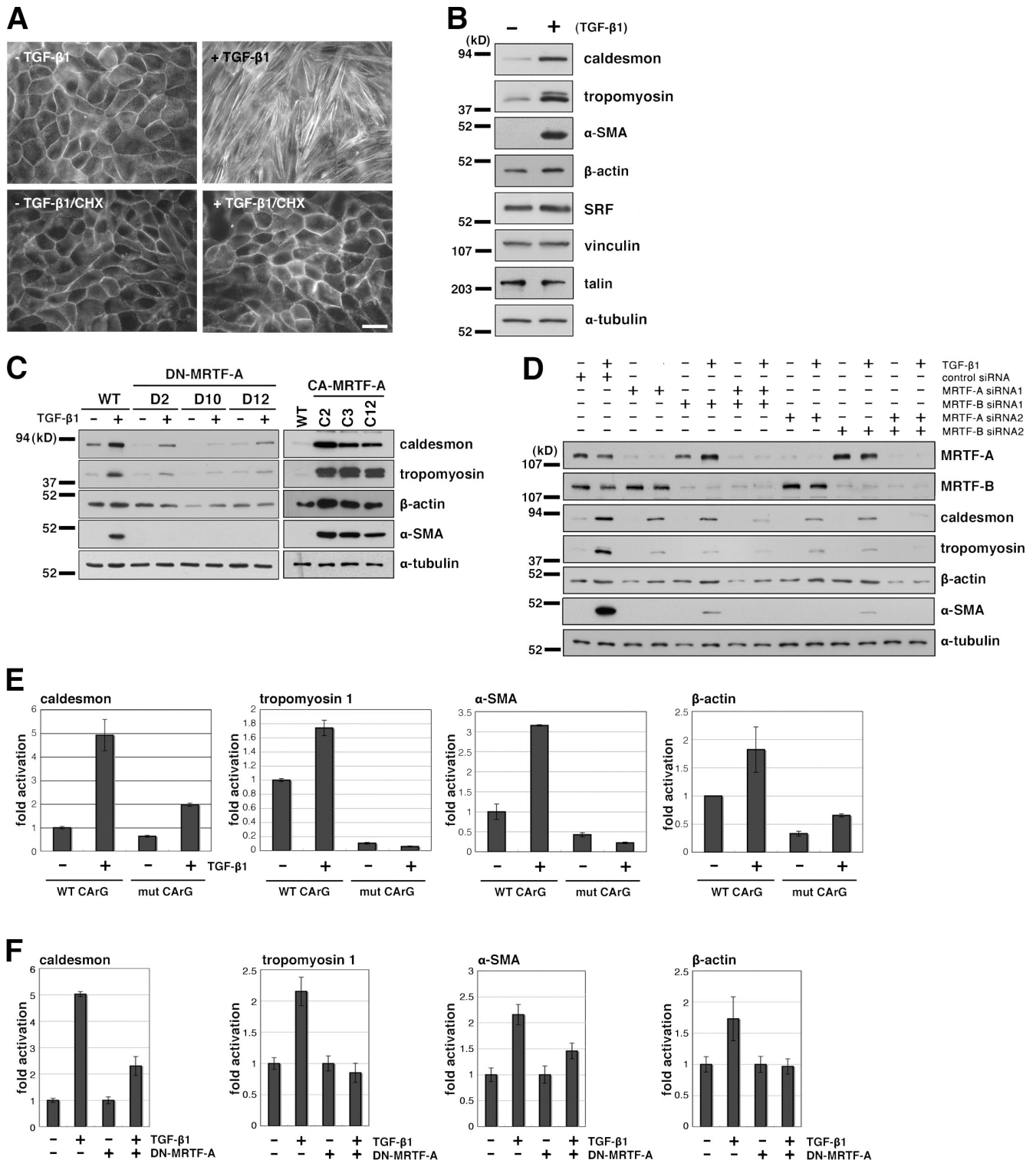
Figure 8. **MRTFs regulate actin remodeling induced by TGF-β1 in MDCK cells.** (A) MDCK cells were cultured with or without TGF-β1 and/or cycloheximide (CHX) for 24 h. Cells were then fixed and stained with Alexa 568–conjugated phalloidin. Bar, 20 μm. (B) MDCK cells were cultured with or without TGF-β1 for 24 h. The expression levels of the indicated cytoskeletal proteins were compared by immunoblotting. (C) DN–MRTF-A (clones D2, D10, and D12) and parental MDCK cells were cultured with or without TGF-β1 (left). CA–MRTF-A (clones C2, C3, and C12) and parental MDCK cells were cultured without TGF-β1 (right). The expression levels of the actin cytoskeletal proteins were compared by immunoblotting. (D) MDCK cells were transfected with MRTF-A or -B siRNAs or control siRNA and cultured with or without TGF-β1. The expression levels of MRTF-A and -B and cytoskeletal proteins were compared by immunoblotting. (E and F) Luciferase reporter assays were performed using promoter constructs for the *caldemon, tropomyosin 1, α-SMA,* and *β-actin* genes that contain a native (WT CArG) or mutated (mut CArG) CArG box (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200708174/DC1). MDCK cells were transfected with these cytoskeletal promoter constructs. 18 h after the transfection, the cells were cultured with or without TGF-β1 for 24 h, and the luciferase activities were measured. For F, the pcDNA3.1–DN–MRTF-A vector was transfected along with the cytoskeletal promoter constructs. Error bars represent SD from three independent experiments.
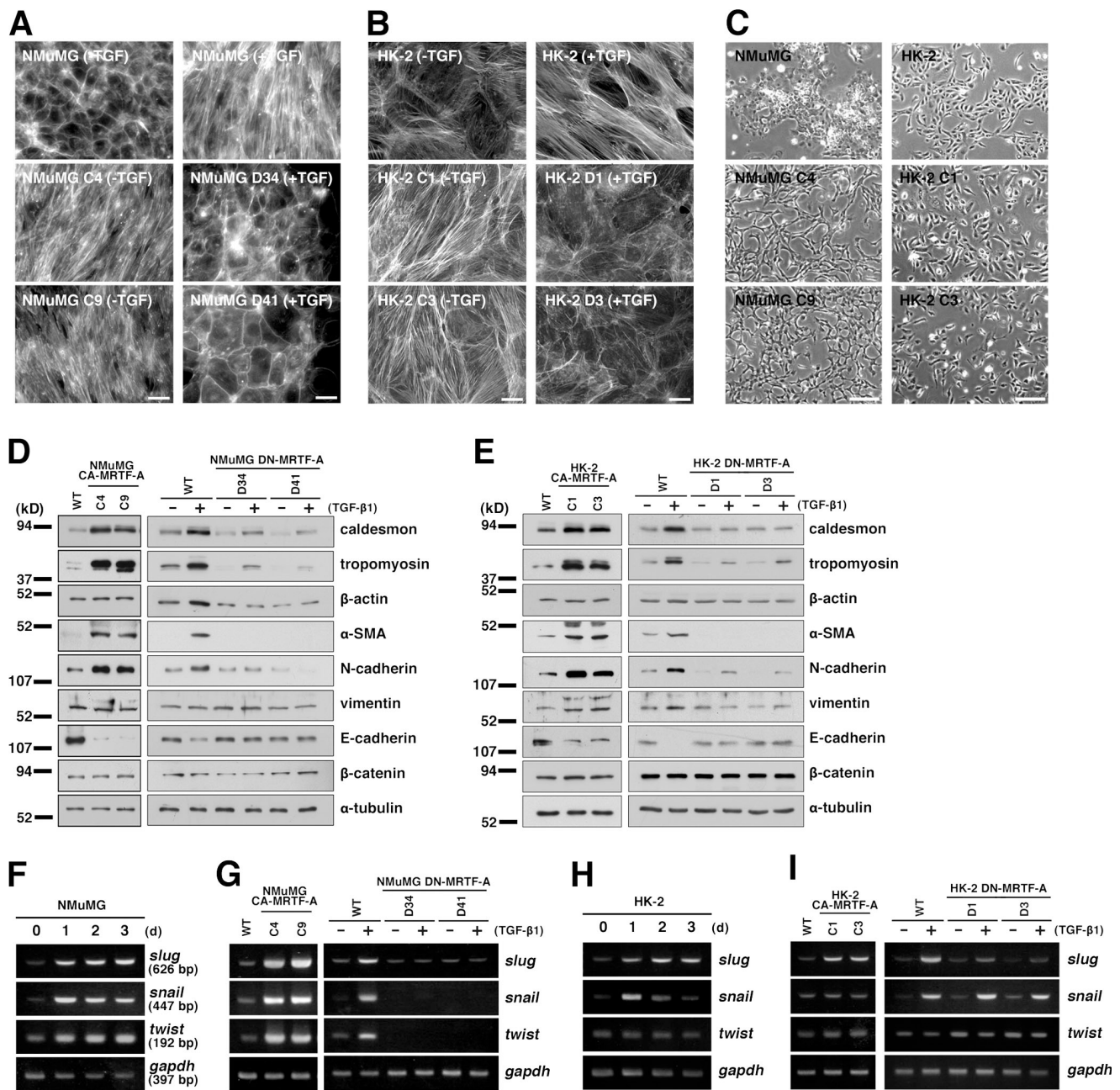
**Figure 9. MRTF-A and -B induce EMT in mouse NMuMG cells and human HK-2 cells.** (A) NMuMG CA–MRTF-A (clones C4 and C9), DN–MRTF-A (clones D34 and D41), and parental NMuMG cells were cultured with or without TGF-β1 for 24 h and stained with Alexa 568–conjugated phalloidin. Bars, 20 μm. (B) HK-2 CA–MRTF-A (clones C1 and C3), DN–MRTF-A (clones D1 and D3), and parental HK-2 cells were cultured with or without TGF-β1 for 48 h and stained with Alexa 568–conjugated phalloidin. Bars, 20 μm. (C) Phase-contrast images of NMuMG CA–MRTF-A (clones C4 and C9) and HK-2 CA–MRTF-A (clones C1 and C3) cells cultured without TGF-β1. Bars, 50 μm. (D and E) NMuMG (WT), NMuMG CA–MRTF-A (clones C4 and C9), HK-2 (WT), and HK-2 CA–MRTF-A (clones C1 and C3) cells were cultured without TGF-β1 (left). NMuMG (WT), NMuMG DN–MRTF-A (clones D34 and D42), HK-2 (WT), and DN–MRTF-A (clones D1 and D3) cells were cultured with or without TGF-β1 for 24 h (NMuMG cell lines) or 48 h (HK-2 cell lines; right). The expression levels of the indicated proteins were compared by immunoblotting. (F) NMuMG cells were cultured with TGF-β1 for the indicated number of days, and the expression time of *slug*, *snail*, and *twist* genes was analyzed by RT-PCR. (G) NMuMG and NMuMG CA–MRTF-A (clones C4 and C9) cells were cultured without TGF-β1 (left). NMuMG and NMuMG DN–MRTF-A (clones D34 and D42) cells were cultured with or without TGF-β1 for 24 h (right). The expression levels of *slug*, *snail*, and *twist* genes were analyzed by RT-PCR. (H) HK-2 cells were cultured with TGF-β1 for the indicated number of days, and the expression time course of *slug*, *snail*, and *twist* genes was analyzed by RT-PCR. (I) HK-2 and HK-2 CA–MRTF-A (clones C1 and C3) cells were cultured without TGF-β1 (left). HK-2 and DN–MRTF-A (clones D1 and D3) cells were cultured with or without TGF-β1 for 24 h (right). The expression levels of *slug*, *snail*, and *twist* genes were analyzed by RT-PCR.

level of *slug* mRNA. In MDCK cells, the expression of *slug*, but not of *snail* or *twist*, was induced by TGF-β1 (Fig. 3, A and B). In MDCK II and HK-2 cells, *slug* and *snail* expressions were

induced by TGF-β1, but the *snail* induction was only transient and greatly diminished 2 d after TGF-β1 stimulation (Figs. 3 B and 9 H). NMuMG cells showed significant induction of all
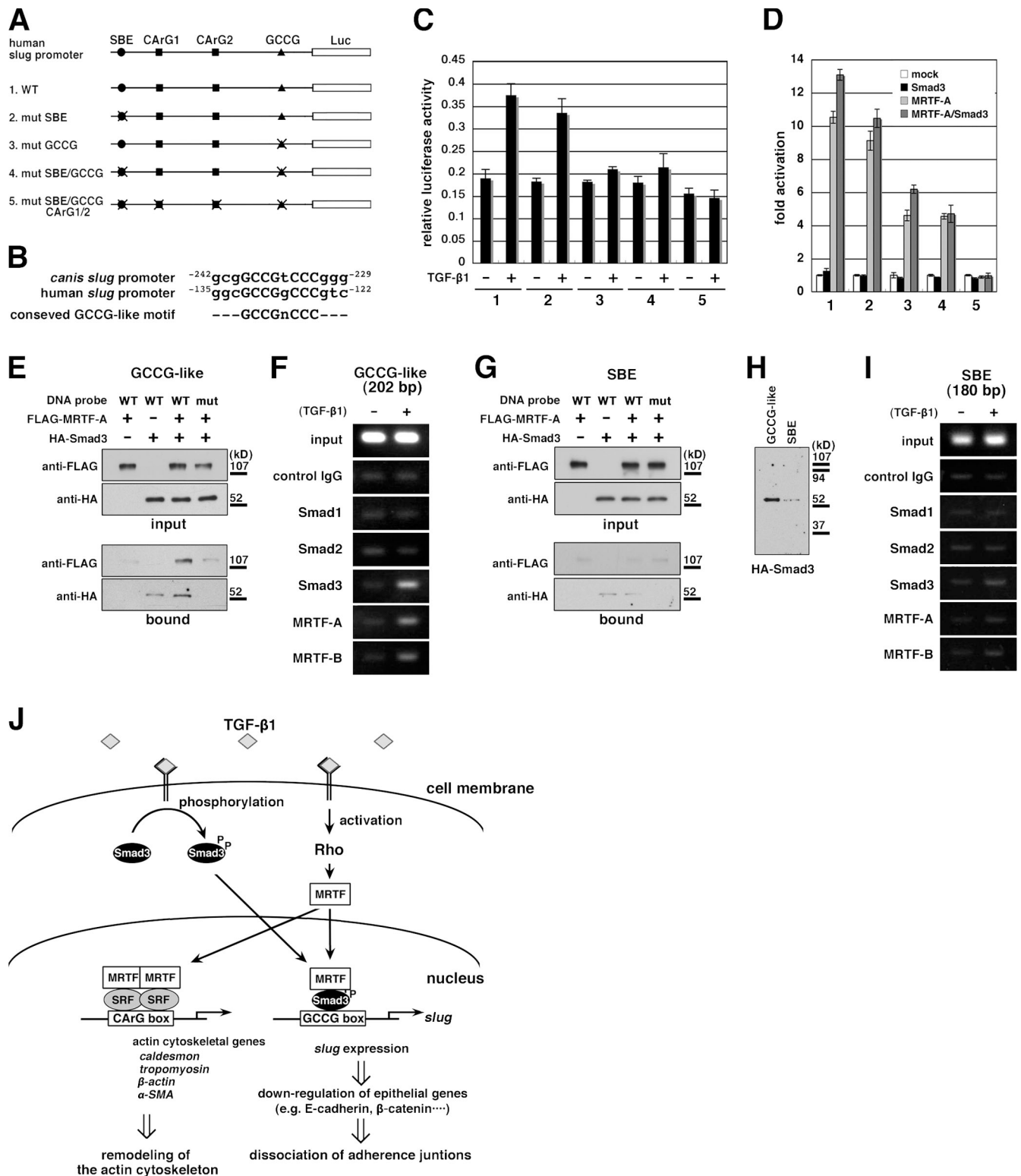
**Figure 10. MRTF/Smad3 also regulates the human *slug* promoter activity.** (A) Schematic representation of human *slug* promoter construct and its site-mutated series. Within the ∼1.1-kb fragment upstream of the first exon, a potential SBE element, CArG box–like sequences, and GCCG-like motif exist. (B) Sequence alignment of GCCG-like motifs within the *C. familiaris slug* and human *slug* promoter regions. The indicated numbers present upstream positions of nucleic acid from start codon. (C) HepG2 cells were transfected with the site-mutated reporter constructs. X-axis numbers indicate the numbers of reporter construct presented in A. The cells were cultured with or without TGF-β1 for 24 h, and then luciferase activities were measured. Error bars represent SD from three independent experiments. (D) HepG2 cells were transfected with the site-mutated reporter constructs along with pcDNA3.1–CA–MRTF-A and/or pcDNA3.1-Smad3(3E). X-axis numbers indicate the numbers of reporter construct presented in A. The luciferase activities were measured 24 h after the transfection. Error bars represent SD from three independent experiments. (E) DNA–protein binding assay was performed using a biotinylated DNA probe containing the human *slug* GCCG-like motif and in vitro–synthesized FLAG–MRTF-A and HA-Smad3. (F) HK-2 cells were cultured with or without TGF-β1

these genes (Fig. 9 F). Thus, the transcriptional regulation of *snail* and *twist* was different in these cell lines, whereas *slug* expression was induced in common by the TGF-β1–MRTF signaling pathway. In *C. familiaris* and human cells, this *slug* induction was regulated by the same transcriptional mechanism via binding of MRTF/Smad3 to the GCCG-like motif within the *slug* promoter (Figs. 7 and 10). In this study, we also suggested the involvement of MRTF in *snail* and *twist* expression. In MDCK cells, *twist* expression was TGF-β1 independent but MRTF dependent (Fig. 3). The basal level of *twist* expression was altered in the MRTF loss- or gain-of-function MDCK clones, which may influence their phenotypes (Fig. 3, C–E). In NMuMG cells, all *slug*, *snail*, and *twist* genes were induced by the TGF-β1–MRTF signaling pathway, even though basal levels of *snail* and *twist* expression were also strongly suppressed by DN–MRTF-A independently of TGF-β1 (Fig. 9 G). In HK-2 cells, transient induction of *snail* by TGF-β1 was MRTF independent. Because the regulatory modes of *snail* and *twist* expressions are complicated, as demonstrated here, further studies are needed to clarify the involvement of MRTFs in their regulations.

All of the myocardin family members (myocardin and MRTF-A and -B) play important roles in muscle differentiation (Wang et al., 2001; Chen et al., 2002; Selvaraj and Prywes, 2003; Li et al., 2005). Myocardin is specifically expressed in cardiac and smooth muscles (Wang et al., 2001), and myocardin-null mice die at embryonic day 10.5 with no evidence of vascular smooth muscle differentiation in the dorsal aorta (Li et al., 2003). Compared with myocardin, MRTF-A and -B are more broadly expressed in various tissues and cells, including nonmuscle cells (Wang et al., 2002). However, their roles, except for the regulation of muscle differentiation, remain largely unknown. Recently, Fan et al. (2007) have reported that contact injury or TGF-β1 stimulation induces the nuclear accumulation of SRF and MRTF-A in Lewis lung carcinoma–PK1 (CL4)–proximal tubular cells. They, however, did not discuss MRTF-A's role in EMT, except for induction of α-SMA expression. We performed the extensive analyses of MRTFs' functions in EMT and revealed that MRTFs play dual roles in EMT: direct regulation of *slug* transcription and reorganization of the actin cytoskeleton. These findings provide novel insights into MRTF's functions in nonmuscle cells. Recently, *MRTF-A* and *-B* knockout mice were reported on (Oh et al., 2005; Li et al., 2006; Sun et al., 2006). Although disruption of the *MRTF-B* gene leads to a lethal defect in pharyngeal arch remodeling, *MRTF-A* knockout mice exhibit a deficit only in the differentiation of mammary myoepithelial cells. In this connection, our present results (Figs. 1 G and 8 D) raise the possibility that MRTF-A and -B in these mice may compensate for one another in the EMT process.

In *D. melanogaster*, whose genome contains only a single member of the myocardin family (MAL-D), MAL-D loss-of-function induces defects in an event similar to EMT: the actin rearrangement in and migration of border cells (Somogyi and Rorth, 2004). This evidence supports the importance of MRTFs in the EMT events.

Smad2 and 3 are major downstream targets of the TGF-β1 pathway. TGF-β1 stimulation immediately induces a nuclear translocation of Smad2/3 via their phosphorylation in a variety of cells (Xu, 2006). Several DNA-binding motifs for Smads have been identified. The GCCG motif (GCCGnCGC) has been identified as a consensus binding sequence for *D. melanogaster* Smad homologue Mad/Medea and for bone morphogenetic protein–regulated Smad1 in mammals, but not for TGF-β–regulated Smad3 (Kusanagi et al., 2000). In our present study, we identified the GCCG-like motif (GCCGnCCC) within the *C. familiaris* and human *slug* promoter region as a TGF-β1–MRTF-responsive element to which the MRTF–Smad3 complex can bind (Figs. 7 and 10). Recent studies using Smad3 knockout mice provide accumulating evidences that Smad3 is required for TGF-β1–induced EMT (Zavadil et al., 2004). In this connection, Hey1 and HMGA2 are identified as direct transcriptional targets of Smad2/3 in TGF-β1–induced EMT (Zavadil et al., 2004; Thuault et al., 2006). Their expressions are immediately and transiently induced 1–8 h after TGF-β1 stimulation, and Hey1 regulates the immediate–early phase of TGF-β1–mediated *snail*, *slug*, and *sip1* inductions in a transient fashion. In our experiments, however, the induction level of *slug* expression was dramatically increased 12–24 h after TGF-β1 stimulation, when the activity of endogenous MRTFs was also increased, and sustained for at least 3 d (Fig. 1 B; and Fig. 3, A and B), suggesting that MRTFs and Hey1/HMGA2 would differently regulate TGF-β1–induced EMT.

This is the first paper to identify MRTFs as EMT regulators with dual functions and to uncover the transcriptional mechanism of the *slug* gene by TGF-β1. Although further studies are needed to clarify the roles of MRTFs in EMT during embryonic development and in fibrosis and tumor progression, our present results provide new insights into the molecular mechanism underlying EMT.

## Materials and methods

for 24 h, and ChIP assay was performed using nonimmune rabbit IgG (control IgG), anti-Smad1, 2, and 3, and anti–MRTF-A and -B antibodies. PCR was performed to determine the human *slug* promoter sequence surrounding the GCCG-like motif. (G) DNA–protein binding assay was performed using a biotinylated DNA probe containing the human *slug* SBE and in vitro–synthesized FLAG–MRTF-A and HA-Smad3. (H) The amounts of HA-Smad3 protein bound to the DNA probes of human *slug* GCCG-like motif or SBE were compared by DNA–protein binding assay. (I) ChIP assay for MRTF-A and -B and Smad1, 2, and 3 bound to the endogenous SBE motif within the human *slug* gene. PCR was performed to determine the human *slug* promoter sequence surrounding the SBE. (J) Scheme of dual functions of MRTFs in TGF-β1–induced EMT. TGF-β1 stimulation induces the phosphorylation and thereby the nuclear translocation of Smad3. TGF-β1 also activates Rho, which results in translocation of MRTFs into the nucleus. The MRTF–Smad3 complex binds to the GCCG-like motif of *slug* gene and enhances *slug* transcription, resulting in the cells becoming scattered in the absence of cell–cell contacts. In addition, the MRTF–SRF complex enhances the transcription of various actin cytoskeletal genes via the CArG box in their promoter regions, leading to actin remodeling.

Cruz Biotechnology, Inc.), antivinculin (HVIN-1; Sigma-Aldrich), anti-talin (8d4; Sigma-Aldrich), anti–E-cadherin (BD Biosciences), anti–N-cadherin (BD Biosciences), antivimentin (Transformation Research), anti–β-catenin (BD Biosciences), anti–α-tubulin (DM1A; Sigma-Aldrich), anti-Smad3 (mouse monoclonal; M05 [Abnova] or rabbit polyclonal [Invitrogen]), anti-HA (3F10; Roche), and anti-FLAG (Sigma-Aldrich) antibodies were purchased. Anti–MRTF-A and -B antibodies were produced in New Zealand rabbits using aa 776–901 of human MRTF-A and aa 913–1061 of human MRTF-B as respective antigens and purified by affinity chromatography using their antigens. Anti-caldesmon antibody was generated as previously described (Tanaka et al., 1993).

### Cell culture
MDCK, MDCK II, NMuMG, and HepG2 cells were cultured in DME supplemented with 10% FCS. HK-2 cells were cultured in DME/F-12 supplemented with 10% FCS. In experiments with TGF-β1, cells were cultured in DME containing 2% horse serum with/without 10 ng/ml of TGF-β1 for 24–48 h.

### Expression vectors and transfection
The coding regions for mouse *MRTF-A* and *-B* and human *Smad1*, *2*, and *3* were amplified by PCR and cloned into the pcDNA3.1(+) expression vector (Invitrogen). CA–MRTF-A (Morita et al., 2007), CA-Smad1 (Smad1(3E); Qin et al., 2001), CA-Smad2 (Smad2(2E); Funaba and Mathews, 2000), CA-Smad3 (Smad3(3E); Chacko et al., 2001), and DN–MRTF-A (Morita et al., 2007) were constructed as previously described. The coding region of the C3 exoenzyme was amplified by PCR using pGEX-KG-TAT-C3 as a template, and then cloned into pcDNA3.1(+). Cells were transfected with these expression vectors using Lipofectamine 2000 (Invitrogen), TransIT-LT1 (Mirus), or nucleofector (Amaxa Biosystems). To establish stable cell lines expressing CA– or DN–MRTF-A, MDCK and NMuMG cells were transfected with pcDNA3.1–CA–MRTF-A or pcDNA3.1–DN–MRTF-A and cultured with 100 μg/ml Geneticin (Invitrogen) to isolate drug-resistant clones. Because HK-2 cells are resistant to Geneticin, HK-2 cell lines expressing CA– or DN–MRTF-A were isolated using Linear Hygromycin Marker (Clontech Laboratories, Inc.).

### Luciferase reporter assay
The promoter regions of human *caldesmon* (Yano et al., 1995), *tropomyosin 1* (Nakamura et al., 2001), $\alpha-SMA$ (Blank et al., 1992), and *β-actin* (Liu et al., 1991) genes were amplified by PCR and cloned into pGL3-basic (Promega; Fig. S3). The promoter regions of the *C. familiaris slug* (available from GenBank/EMBL/DDBJ under accession no. AB300658) and human *slug* (available from GenBank/EMBL/DDBJ under accession no. AB300659) genes were amplified by PCR and cloned into pGL3-basic. To construct the 9xcore region–Luc vector, the core region sequence (5′-tcgcggccgtccc-gggggcggggctg-3′) was synthesized in vitro, and nine copies of the core region fragments were tandemly inserted into pGL3-basic. The 3xCArG-Luc vector was constructed with reference to a previous paper (Hill et al., 1994). In brief, three tandem repeats of the *c-fos* CArG box sequence (5′-attggatgtccatattaggacatct-3′) were synthesized in vitro as a single unit. The TATA box sequence of the *Xenopus γ-actin* gene was amplified by PCR and inserted into pGL3-basic together with the synthesized 3xCArG oligonucleotide. These constructs were introduced into MDCK or HepG2 cells along with pSV-βGal (Promega), which was used to normalize the transfection efficiency. 24 h after the transfection, the cells were lysed with Passive lysis buffer (Promega), and the luciferase and β-galactosidase activities were measured using the luciferase assay system (Promega) and Luminescent β-galactosidase detection kit II (Clontech Laboratories, Inc.), respectively.

### Immunocytochemistry and microscopy
Cells grown on coverslips were fixed using 4% paraformaldehyde in PBS for 30 min at room temperature, and then permeabilized with 0.1% Triton X-100 in PBS. The fixed cells were incubated with the primary antibody, followed by the appropriate Alexa 488–conjugated secondary antibodies (Invitrogen). To visualize actin filaments or the nuclei, Alexa 568–conjugated phalloidin (Invitrogen) or Hoechst 33342 (Invitrogen) was added with the secondary antibody solution. The stained cells were mounted in GEL/MOUNT mounting medium (Biomeda) and observed using a microscope (IX70; Olympus) with a 60×/1.25 lens (UPlanFLN; Oil Iris; Olympus) at room temperature. Fluorescent images were acquired with a camera (CoolSNAP HQ CCD; Photometrics) using MetaMorph software (MDS Analytical Technologies) and processed with Photoshop CS software (Adobe).

### RT-PCR
Total RNA was extracted from cells using TRIzol reagent (Invitrogen) and reverse transcribed using PrimeScript Reverse Transcriptase (Takara Bio Inc.).

The cDNA was amplified using the following PCR primers: *slug*, 5′-atgccgc-gctccttcctg-3′/5′-gtgtgagttctaatgtgtccttgaag-3′; *snail*, 5′-accttccagcagc-cctacagc-3′/5′-gcagcgtgtggcttcggatg-3′; *twist*, 5′-cctcggayaagctgagcaa-gat-3′/5′-ctagtgggacgcggacatgga-3′; and *gapdh*, 5′-tcttcaccaccatggaga-agg-3′/5′-gaaggccatgccagtgag-3′.

### Coimmunoprecipitation
Cells were lysed with lysis buffer (1 mM EDTA, 0.1% Triton X-100, 50 mM NaF, 1 mM Na$_3$VO$_4$, 1 mM β-glycerophosphate, and protease inhibitor cocktail tablets [Roche] in PBS, pH 7.4, [Nakarai Tesque Inc.]) and incubated on ice for 15 min. After a brief sonication, the lysates were spun at 10,000 *g* for 30 min to remove cell debris. The resulting supernatants were incubated overnight (16–24 h) with the indicated antibodies. After this incubation, protein G–Sepharose (GE Healthcare) was added to the mixtures, which were further incubated for 4 h with gentle agitation. To elute the immunocomplexes, Sepharose beads were washed four times with the lysis buffer, and then SDS sample buffer was added. To examine the interaction between MRTFs and Smad3, MRTF-A and -B, Smad3, and the products of their corresponding deletion constructs were synthesized in vitro using TNT Quick Coupled Transcription/Translocation Systems (Promega). The synthesized proteins were mixed in the lysis buffer in various combinations and their interactions were evaluated by coimmunoprecipitation.

### RNA interference
Cells were transfected with siRNA using Lipofectamine RNAiMAX (Invitrogen) and cultured for 2 d before analysis. The siRNA sequences were as follows: *C. familiaris* MRTF-A siRNA1, 5′-caggtgaattatccgaaagta-3′; *C. familiaris* MRTF-A siRNA2, 5′-ctgcgtgcatatcaagaacaa-3′; *C. familiaris* MRTF-B siRNA1, 5′-cccaagaatcctaatgacaaa-3′; *C. familiaris* MRTF-B siRNA2, 5′-accaatgaaattgttccacta-3′; human MRTF-A siRNA1, 5′-gacacctcggaattg-cacttt-3′; human MRTF-A siRNA2, 5′-cccgagtcagcgaaccactgc-3′; human MRTF-B siRNA1, 5′-ggacgagagaacaactagtgg-3′; human MRTF-B siRNA2, 5′-gtatgctggaccattcacact-3′; human Smad3 siRNA1, 5′-atcaagggatttcctat-ggaa-3′; and human Smad3 siRNA2, 5′-aagagattcgaatgacggtaa-3′. In control experiments, scrambled siRNA (Santa Cruz Biotechnology, Inc.) was used.

### DNA–protein binding assay
In vitro–synthesized FLAG–MRTF-A and/or HA-Smad3 proteins were diluted with 500 μl of DNA affinity precipitation buffer (20 mM Hepes-KOH, pH 7.9, 80 mM KCl, 1 mM MgCl2, 0.2 mM EDTA, 0.5 mM DTT, 10% wt/vol glycerol, and 0.1% Triton X-100) and incubated with 1 μg of biotinylated double-stranded DNA probe and 15 μg of herring sperm DNA for 1.5 h at 4°C. In competition experiments, 50 μg of nonbiotinylated DNA probe or nonbiotinylated mutated DNA probe was also added. Dynabeads M-280 Streptavidin (Invitrogen) was then added to the DNA–protein mixture, and the sample was gently mixed by rotation for 1.5 h at room temperature. The beads were washed with DNA affinity precipitation buffer, and the bound proteins were eluted with SDS sample buffer. The sequences of DNA probes were as follows: core region of *C. familiaris slug* promoter, 5′- gttcgcg-gccgtccggggcggggctgaaa-3′; mutated core region of *C. familiaris slug* promoter, 5′-gttcgcgaaagtcccggggcggggctgaaa-3′; GCCG-like motif of human *slug* promoter, 5′-tcagaggcgccggcccgtccgtct-3′; mutated GCCG-like motif of human *slug* promoter, 5′-tcagaggcaaaggcccgtccgtct-3′; SBE of human *slug* promoter, 5′-aaataattgtctctaaagacccatacaa-3′; and mutated SBE of human *slug* promoter, 5′-aaataattatatctaaaaaaccatacaa-3′.

### ChIP assay
MDCK or HK-2 cells cultured in DME containing 2% horse serum with or without TGF-β1 for 24 h were used in a ChIP assay performed with a ChIP assay kit (Millipore) and anti–MRTF-A and -B and anti-Smad1, 2, and 3 antibodies. The sequences of primers used for the PCR amplification were as follows: *C. familiaris slug* core region, 5′-ccgtcctgcgccgcagcgg-3′ and 5′-ctcctgcgccgactgagcg-3′; human *slug* GCCG-like motif, 5′-cctctcagctgt-gattggatc-3′ and 5′-acggcggtccctacagcatc-3′; and human SBE, 5′-gaaact-ggtgagatactgagatgg-3′ and 5′-gaaccaccggacattctctc-3′.

### Online supplemental material
Fig. S1 shows a phenotype of MDCK cell lines expressing CA–MRTF-A (clones C3 and C12) or DN–MRTF-A (clones C10 and C12). Fig. S2 shows the full sequence of the *C. familiaris slug* promoter region used in this paper. Fig. S3 shows full sequences of promoter regions of the actin cytoskeletal genes used in this paper. Fig. S4 shows the nuclear translocation of MRTF-A and -B in response to TGF-β1 stimulation in NMuMG and HK-2 cells. Fig. S5 shows the full sequence of the human slug promoter region

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