Integrin α3β1–dependent β-catenin phosphorylation links epithelial Smad signaling to cell contacts

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Injury-initiated epithelial to mesenchymal transition (EMT) depends on contextual signals from the extracellular matrix, suggesting a role for integrin signaling. Primary epithelial cells deficient in their prominent laminin receptor, α3β1, were found to have a markedly blunted EMT response to TGF-β1. A mechanism for this defect was explored in α3-null cells reconstituted with wild-type (wt) α3 or point mutants unable to engage laminin 5 (G163A) or epithelial cadherin (E-cadherin; H245A). After TGF-β1 stimulation, wt epithelial cells but not cells expressing the H245A mutant internalize complexes of E-cadherin and TGF-β1 receptors, generate phospho-Smad2 (p-Smad2)–pY654–β-catenin complexes, and up-regulate mesenchymal target genes. Although Smad2 phosphorylation is normal, p-Smad2–pY654–β-catenin complexes do not form in the absence of α3 or when α3β1 is mainly engaged on laminin 5 or E-cadherin in adherens junctions, leading to attenuated EMT. These findings demonstrate that α3β1 coordinates cross talk between β-catenin and Smad signaling pathways as a function of extracellular contact cues and thereby regulates responses to TGF-β1 activation.

Introduction

Integrins, a group of heterodimeric surface molecules, provide a dynamic interface between the cell and ECM by regulating inside-out and outside-in signaling during embryonic development, wound repair, and tissue morphogenesis (Geiger et al., 2001; Hynes, 2002). Epithelial integrins play an important role in the process of differentiation and homeostasis of skin, kidney, and lung lining layers (Kreidberg et al., 1996). One integrin highly expressed in epithelial cells, α3β1, mediates cell–matrix interactions with its major ligand laminin 5 (Ln5; Eble et al., 1998) and localizes to focal adhesion sites (Dogic et al., 1998). In addition, α3β1 is also found along lateral cell borders in association with epithelial cadherin (E-cadherin) in adherens junctions (Nakamura et al., 1995; Chattopadhyay et al., 2003), where its colocalization is thought to contribute to assembly and maintenance of cell–cell contacts and normal barrier function (Wang et al., 1999; Lubman et al., 2000). Whether α3β1 has a structural role in adherens junction maintenance or acts in some way to sense perturbations in cell–cell contacts is unknown.

Under certain conditions, epithelial cells undergo the process of epithelial to mesenchymal transition (EMT). EMT involves loss of epithelial characteristics by down-regulation of proteins such as E-cadherin and a shift toward a fibroblast-like phenotype with expression of mesenchymal proteins such as α smooth muscle actin (α-SMA), matrix metalloproteinases (MMPs), and enhanced motility (Savagner, 2001; Kalluri and Neilson, 2003). The most well-studied cytokine as an inducer of EMT is TGF-β1 (Gotzmann et al., 2004; Zeisberg and Kalluri, 2004). TGF-β1 binds to its receptor, through which it activates intracellular Smad-dependent and -independent signaling pathways and regulates gene transcription and cell shape (Derynck and Zhang, 2003).

TGF-β1 signaling is regulated at multiple levels beginning with activation of latent TGF-β1 itself, determinants of phosphorylation of receptor-associated Smads (i.e., Smads 2 and 3) by the TGF-β1 receptor, and various factors that influence stability and translocation of phospho-Smads (p-Smads) to the nucleus. Smad-dependent transcription is further dependent on coactivators that stabilize Smad association with DNA, and these coregulators are both constitutive, such as p300, and cell

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Abbreviations used in this paper: α-SMA, α smooth muscle actin; AEC, alveolar epithelial cell; E-cadherin, epithelial cadherin; EMT, epithelial to mesenchymal transition; ERK, extracellular signal-regulated kinase; Fn, fibronectin; IP, immunoprecipitation; MDC, monodansylcadaverine; MMP, matrix metalloproteinase; p-Smad, phospho-Smad; shRNA, short hairpin RNA; wt, wild type.

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type specific. As a consequence of this complexity, multiple signaling molecules are known to modulate TGF-β1 signaling during EMT and tumorigenesis, including bone morphogenetic proteins and their receptors as well as Ras, MAPK, and various cytokines (Massague and Chen, 2000).

There is circumstantial evidence that integrins via their engagement with the ECM play a substantial role in TGF-β1 activation and signaling. Integrin αVβ6 and binding to the ECM molecule fibronectin (Fn) is required for activation of latent TGF-β1 (Fontana et al., 2005; Sheppard, 2005). Global inhibition of β1-integrin matrix binding by blocking antibodies is reported to abrogate active TGF-β1-mediated p38 MAPK activation and EMT in mammary cells (Bhowmick et al., 2001). Attachment of epithelial cells to various ECMs is known in some way to promote responses to active TGF-β1 (Thannickal et al., 2003). Several prior studies implicate the β1 integrin–associated protein, integrin-linked kinase in EMT, also implying an important role for β1 integrins in the process (Lee et al., 2004; Oloumi et al., 2004). However, to date, there is no specific integrin α chain associated with β1 that is known to be critically involved in EMT, and the molecular mechanisms by which ECM regulates EMT are undefined.

The impetus for this study was the observation that an immortalized α3-null kidney epithelial cell line failed to up-regulate the expected mesenchymal markers after TGF-β1 stimulation. We extended this observation by finding defective TGF-β1 responses in primary lung epithelial cells selectively missing α3β1. We have used the α3-null cell line to explore the mechanisms by which α3β1 influences cellular responses to TGF-β1. These experiments reveal not only an unrecognized role for α3β1 in EMT but also a molecular mechanism that can explain how cell contacts critically influence responses to TGF-β1 signaling.

Results

Primary lung alveolar epithelial cells (AECs) deprived of α3β1 show an impaired response to TGF-β1

Primary AECs isolated from a conditional α3-null mouse were exposed to adenovirus-Cre for 96 h to abrogate integrin α3 expression. As a control, cells were infected in parallel with a GFP-expressing adenovirus. Cells were maintained on Matrigel/collagen because on this matrix the cells activate little TGF-β1 and maintain an epithelial phenotype (Kim et al., 2006). At the end of 96 h, the primary cells were harvested and transferred to Fn-coated surfaces for an additional 4 d to allow for TGF-β1 activation and initiation of EMT, as previously reported (Kim et al., 2006). This point is illustrated in Fig. 1 A (left), showing phosphorylation of Smad2 after 4 d on Fn and induction of α-SMA. Inhibition of TGF-β1 receptor kinase activity with SB431542 blocked p-Smad2 formation and also attenuated up-regulation of α-SMA. Immunoblotting indicated a near-complete absence of α3 in adenovirus-Cre–treated cells as compared with control cells bearing the unrecombined floxed α3 allele (Fig. 1 A, right) and a marked decrease in TGF-β1–induced expression of α-SMA and collagen I in α3β1-null cells. The presence or absence of α3 had no effect on Fn-induced TGF-β1 activation, as judged by equivalent p-Smad2 levels (Fig. 1 A, right). These findings were confirmed in experiments of primary AECs derived from mice with lung epithelial–specific loss of α3 achieved by crossing $\alpha3^{fl/fl}$ mice with mice carrying the human *SPC-rtTA* (surfactant protein C promoter–reverse tetracycline-controlled transactivator) and *tetO-Cre* (tetO-cytomegalovirus-Cre) transgenes (Kim et al., 2008). The phenotypes of these mice, termed FASC (floxed α3 *SPC-rtTA tetO-Cre*) mice, are the subject of a separate study (Kim et al., 2008). AECs isolated from FASC mice also demonstrated a markedly impaired EMT response to Fn ex vivo (unpublished data). Thus, epithelial cell α3 appears critical for up-regulation of mesenchymal markers in response to endogenous TGF-β1 activation. We next undertook a series of experiments to explore the molecular mechanisms underlying the role of this integrin in TGF-β1 signaling.

α3β1 regulates kidney epithelial cell responses to TGF-β1

To assess the specific role of α3β1, we used immortalized kidney $\alpha3^{-/-}$ epithelial cells (Kreidberg et al., 1996). The basal morphology of these cells on laminin (Matrigel), serum-coated surfaces, and Fn was found to be similar. Unlike primary AECs, this cell line did not activate Smads upon attachment to Fn and maintained a clustered phenotype, allowing us to separate determinants of the activation of TGF-β1 from cellular responses to active TGF-β1. We reintroduced into cells wild-type (wt) α3 or α3 head domain point mutants previously shown to block the interaction of α3β1 with Ln5 (G163A) or its cis-acting ligand, uPAR (urokinase receptor; H245A; Zhang et al., 1999, 2003). Surface expression detected by FACS and total protein by Western blotting of both wt α3 and the mutants were comparable with and similar to endogenous α3 in mouse kidney epithelial cells (Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200806067/DC1). We initially exposed $\alpha3^{-/-}$ cells and cells reconstituted with the various integrins to active TGF-β1 for 48 h and observed their phenotype (Fig. 1 B). TGF-β1 induced striking declustering and scattering in wt α3– and G163A-expressing cells, whereas α3-null and H245A mutant cells appeared unaffected. Time-lapse microscopy indicated that wt cells began to scatter at ∼18 h after exposure to active TGF-β1, whereas H245A cells never underwent a scattering response at >48 h of observation (Videos 1 and 2). Further stimulation time up to 96 h did not alter the observed pattern (unpublished data). The G163A mutation mimicked the wt phenotype changes but responded to TGF-β1 faster, suggesting that the capacity of the integrin to engage Ln5 may not be critical to TGF-β1 signaling. The $\alpha3^{-/-}$ cells showed membrane ruffling; however, declustering was more difficult to assess because these cells do not form extensive cell–cell contacts at baseline, as previously reported (Wang et al., 1999). Enhanced scattering was not observed (Fig. 1 B). Two independent clones each of α3 wt, H245A, and G163A cells showed the same phenotypic response to TGF-β1. Most subsequent experiments were performed on serum-coated surfaces because for cells exhibiting a morphological response to TGF-β1, the responses appeared to be qualitatively similar on serum- or Fn-coated surfaces.

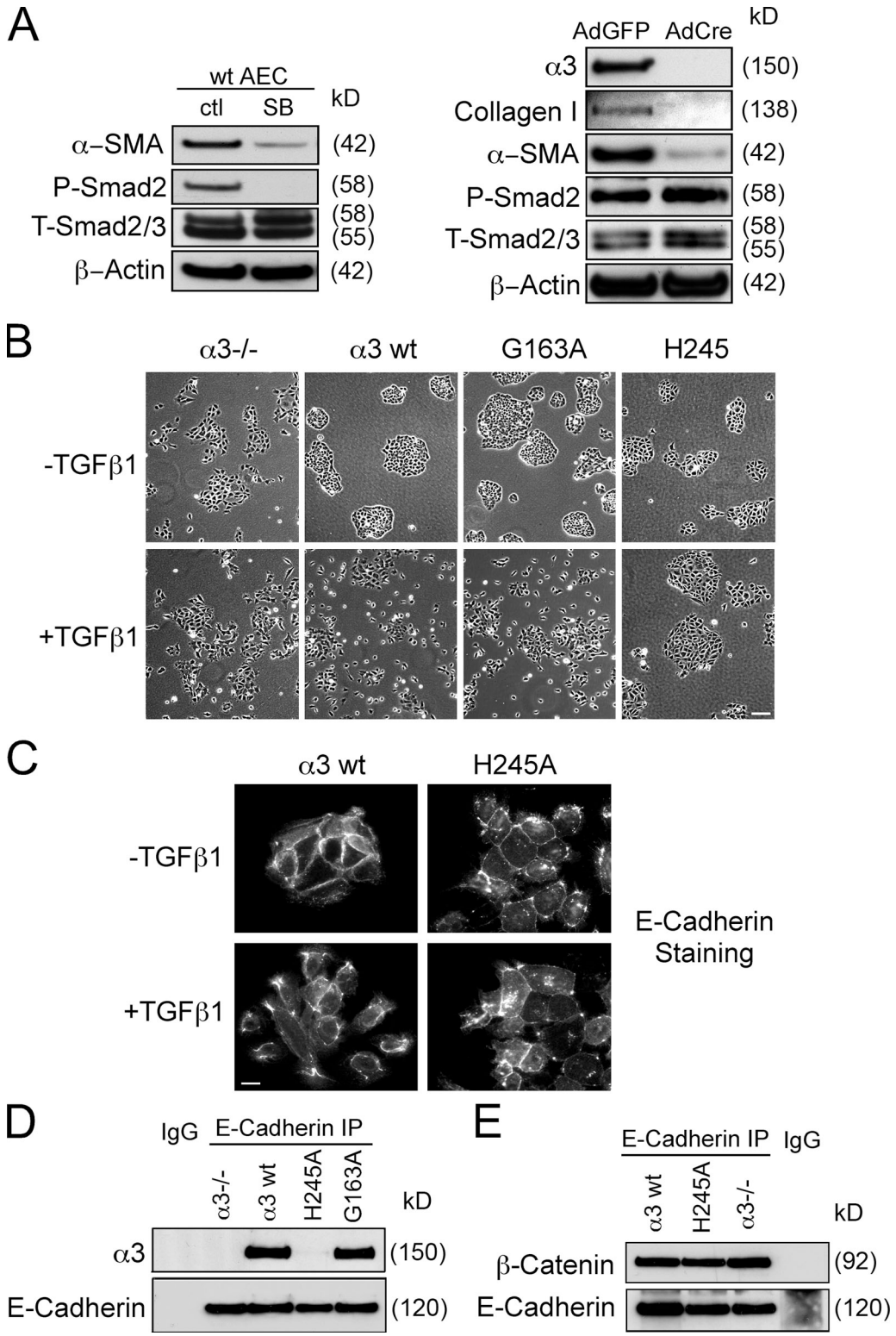

Figure 1. **TGF-β1 responses correlate with α3β1 association with E-cadherin but not Ln5.** (A) TGF-β1 responses in primary AECs from wt (left) or α3 conditional null mice (right). wt AECs were cultured on Fn to induce endogenous TGF-β1 activation and were incubated with TGF-β1 receptor kinase inhibitor SB431542 (SB) or DMSO (ctl) for 4 d. α3 conditional AECs were initially cultured on Matrigel/collagen matrices and treated with either adeno-Cre (AdCre) virus or 20 plaque-forming U/cell adeno-GFP control virus. After 96 h, cells were replated on Fn for 4 d. Cell extracts were blotted for various proteins. SB431542 blocks Smad2 phosphorylation and the α-SMA response in wt AECs. Loss of α3 does not affect Smad2 phosphorylation but blocks up-regulation of α-SMA and collagen I. (B) Phase photographs of $\alpha3^{-/-}$, α3 wt, H245A, and G163A mutant cells at baseline and after 48-h stimulation with TGF-β1. Videos showing phenotypic change and cell motility of α3 wt and H245A cells after TGF-β1 stimulation are provided (Videos 1 and 2, available at http://www.jcb.org/cgi/content/full/jcb.200806067/DC1). Bar, 50 μm. (C) E-cadherin distribution in α3 wt and H245A mutant cells without and with 48-h TGF-β1 stimulation. Bar, 1 μm. (D) E-cadherin coimmunoprecipitates with wt α3– and G163A-expressing cells but does not coimmunoprecipitate with the H245A mutant. (E) E-cadherin–β-catenin association is not affected by α3β1. The aforementioned experiments have been performed at least three times with similar results.

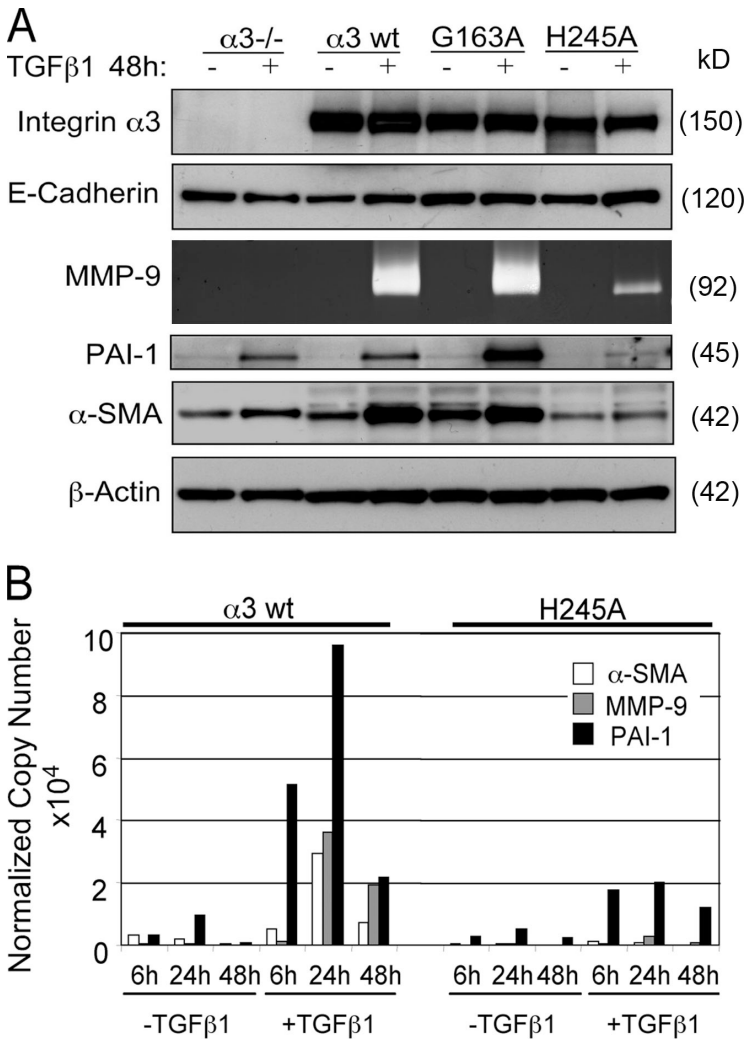

Figure 2. **TGF-β1 up-regulates the expression of MMP-9, PAI-1, and α-SMA in α3 wt cells but not in H245A mutant cells.** (A) TGF-β1 induces expression of MMP-9, PAI-1, and α-SMA in α3 wt and G163A mutant cells but not in H245A mutant cells. Serum-starved cells were stimulated with 4 ng/ml TGF-β1 for 48 h. Supernatants were collected and concentrated 10 times. Cells were lysed in RIPA buffer and blotted for integrin α3, E-cadherin, PAI-1, α-SMA, and β-actin. MMP-9 was detected by gelatin zymography. The aforementioned blots have been repeated at least three times with a similar pattern. (B) Time course of mRNA expression in α3 wt and H245A cells stimulated with TGF-β1. Serum-starved cells were stimulated with 4 ng/ml TGF-β1 for different time periods. RNA was extracted from the cells and used for direct Taqman real-time PCR analysis. Data shown are representative of four independent experiments.

Note that E-cadherin was not down-regulated as judged by total protein in any of the cells after 48 h (Fig. 2 A), which is consistent with prior evidence that suppression of E-cadherin expression during EMT often requires 7–14 d (Thiery and Sleeman, 2006). However, the distribution of E-cadherin on the surface of epithelial cells was strikingly different among the cells at baseline and after 48-h TGF-β1 stimulation. As previously reported, α3-null cells have less surface E-cadherin even though total protein is equivalent to that of wt cells, indicating that surface expression is very unstable and most of the E-cadherin at steady state is contained in endosomal vesicles (Wang et al., 1999). This likely accounts for the scattered appearance of null cells in culture at baseline (Fig. 1 B). In contrast, cells expressing either wt or mutant α3 integrins have comparable surface expression of E-cadherin and form extensive cell–cell contacts at baseline (Fig. 1 C). TGF-β1 treatment for 48 h results in a marked shift of E-cadherin away from cell–cell contacts and into the necks of lamellipodia of wt (or G163A mutant) cells but had little or no apparent effect on H245A-expressing cells (Fig. 1 C).

The lack of change in E-cadherin distribution after TGF-β1 stimulation in H245A-expressing cells raised the possibility that the integrin associates with and affects trafficking of E-cadherin. Chattopadhyay et al. (2003) previously reported that α3β1 formed complexes with CD151 and E-cadherin, although no role for this complex in growth factor signaling or trafficking of E-cadherin was investigated. Indeed, α3 wt and G163A integrins were robustly coimmunoprecipitated with E-cadherin, whereas the H245A mutant was undetectable in E-cadherin immunoprecipitates (Fig. 1 D). It is noteworthy that β-catenin, known to associate with the cytoplasmic tails of E-cadherin, coprecipitated equally with E-cadherin among all of the cell lines examined (Fig. 1 E). Thus, there is a strong correlation between the capacity of α3β1 to associate with E-cadherin and the cellular responses to TGF-β1.

To further examine the obvious differences among the various cells after TGF-β1 stimulation, we looked for changes in protein expression of known TGF-β1 target genes. Western blot analysis and gel zymography confirmed that several such proteins could be reliably detected: plasminogen activator inhibitor-1 (PAI-1), MMP-9, and α-SMA. As expected, wt α3– and G163A-bearing cells strongly up-regulated MMP-9, PAI-1, and α-SMA after 48-h TGF-β1 stimulation, whereas H245A and $\alpha3^{-/-}$ cells showed either partial or completely absent responses of MMP-9 and α-SMA (Fig. 2 A). PAI-1 was completely suppressed in H245A-expressing cells, but α3-null cells continued to respond, indicating some difference in the

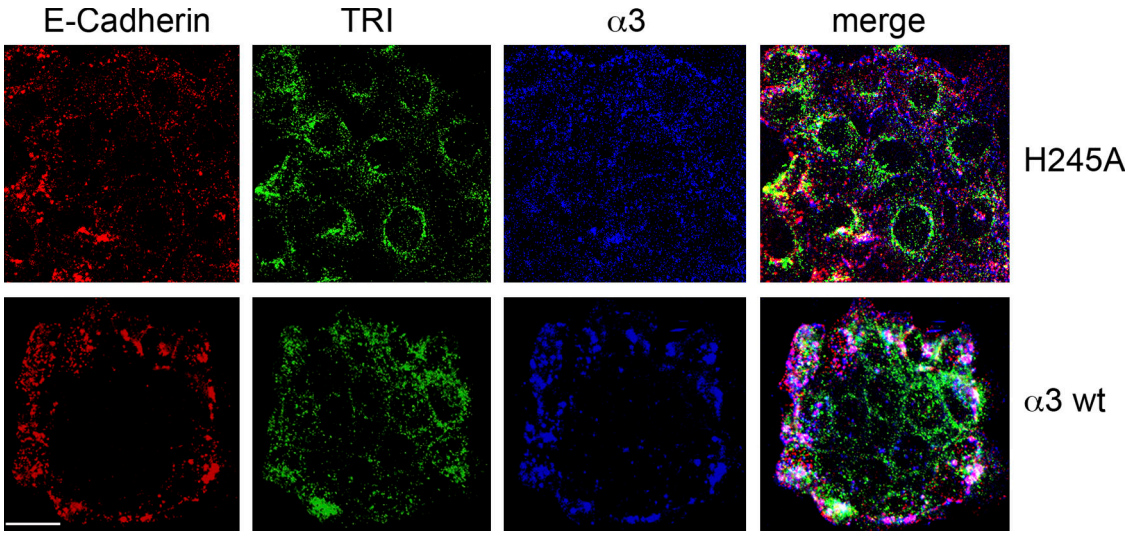

Figure 3. **Identification of colocalized α3β1, E-cadherin, and TGF-βR1 on cell surfaces of α3 wt and H245A mutant cells.** Cells serum starved overnight were exposed to 300 μM MDC to inhibit clathrin-mediated endocytosis for 30 min and TGF-β1 for 4 h. E-cadherin and α3β1 were clustered with fluorescent secondary antibodies on live cells, and the cells were fixed and stained for TGF-βR1 (TRI). Representative confocal images with individual color and the merged images of α3 wt and H245A mutant cells are shown. Quantitative analysis of E-cadherin–α3β1–TGF-βR1 coclusters comparing multiple fields on each of six separate clusters for each cell type indicated significantly ($P < 0.001$) more colocalization on α3 wt cells (see Identification of a tripartite complex…. for details). This experiment has been performed three times with similar results. Bar, 2 μm.

requirements for induction of PAI-1 compared with MMP-9 and α-SMA in these cells.

We compared protein levels and corresponding mRNA levels in α3 wt and H245A mutant cells at different time points after TGF-β1 stimulation. α3 wt cell mRNAs for MMP-9, PAI-1, and α-SMA peak at 24 h of stimulation and then wane (Fig. 2 B). H245A follows a similar time course; however, expression levels are strongly decreased compared with the wt. These results are consistent with the time course of TGF-β1 responses at the protein level (Fig. S1 B). We next turned to whether the differences in TGF-β1 responses among the various cell lines could be explained by altered Smad phosphorylation and/or nuclear translocation.

Smad phosphorylation, nuclear translocation, and MAPK activation are α3 integrin independent

The striking differences in mRNA responses were not mirrored by changes in Smad phosphorylation (Fig. S2 A, available at http://www.jcb.org/cgi/content/full/jcb.200806067/DC1). Strong TGF-β1–dependent Smad2 phosphorylation and nuclear accumulation was seen at the 15-min time point as described by other groups (Nakao et al., 1997; Shen et al., 1998) and revealed no difference between α3 wt integrin and the mutants. Fractionation was confirmed by a lack of β-actin in the nuclear immunoblots. Separate experiments comparing wt and α3-null cells also showed no differences in Smad2 phosphorylation (unpublished data). Smad7 protein was detectable in both wt and H245A mutant cells but was found to not be different either at baseline or after TGF-β1 exposure (unpublished data). To further test this point, we transiently transfected α3 wt and H245A mutant cells with a luciferase construct containing 12 repeats of the Smad3/Smad4-binding element CAGA (Dennler et al., 1998) and measured transcriptional activity after TGF-β1 stimulation (Fig. S2 B). Both the α3 wt and H245A mutants respond to TGF-β1 with an ∼10-fold increase in the CAGA promoter activity, indicating that Smad functionality in the nucleus is not impaired in the H245A mutant.

We next considered Smad-independent TGF-β1 signaling pathways known to be influenced by integrins. TGF-β1 affects the MAPK cascades via Ras to extracellular signal-regulated kinase (ERK) and via TGF-β–activated kinase 1 to JNK and p38, modulating TGF-β1–mediated transcription (Derynck and Zhang, 2003). Because MAPKs also integrate downstream signals derived from integrins, we asked whether the signaling cascades through ERK, JNK, and p38 were activated differentially among the cells expressing the various forms of α3β1. Although ERK phosphorylation increased transiently after TGF-β1 stimulation in all of the cell lines, inhibitors of MAPK kinase (PD98059), p38 (SB202190), and JNK (JNK-II inhibitor) over a wide concentration range did not affect the measured TGF-β1–mediated transcriptional responses in α3 wt or H245A mutant cells (unpublished data). However, these experiments did confirm that motility responses of the cells and transcriptional responses can be dissociated, as has been previously reported (Ozdamar et al., 2005; Wang et al., 2005). The 50–500-nM JNK-II inhibitor completely blocked α3 wt cell scattering after TGF-β1 in a dose-dependent matter, but the induction of PAI-1, α-SMA, and MMP-9 was not affected by the JNK-II inhibitor (Fig. S3, available at http://www.jcb.org/cgi/content/full/jcb.200806067/DC1), suggesting that the TGF-β1 signal divided up proximal of the JNK MAPK and that the integrin influence on TGF-β1 signaling occurs proximally in a signaling cascade. We also tested an inhibitor of glycogen synthase kinase 3β (GSK3β; TPK I inhibitor), which is the kinase known to influence β-catenin signaling and previously linked to β1-integrin signaling. The inhibitor did not affect induction of the mesenchymal markers in these cells (unpublished data). Thus, we could

not readily ascribe the marked functional differences in cellular responses to TGF-β1 affected by α3β1 to differences in MAPK or GSK3β signaling.

Identification of a tripartite complex of E-cadherin, α3β1, and TGF-βR1

Because cellular responses to TGF-β1 strongly correlated with the capacity of integrin α3β1 to associate with E-cadherin (Fig. 1), we considered the possibility that E-cadherin, α3β1, and TGF-β1 receptors all physically interact. Preliminary attempts to visualize E-cadherin–TGF-βR1 complexes on the cell surface indicated that little or no TGF-βR1 could be found enriched in adherens junctions. However, when E-cadherin (Fig. 3, red) was clustered on live cells expressing wt α3, a fraction of α3 (Fig. 3, blue) was found to cocluster (Fig. 3, purple) on the apical surfaces. The degree of coclustering appeared to depend on the degree of E-cadherin available for clustering, mainly around the edges of cellular islands and distinct from adherens junctions. Likewise, colocalization of E-cadherin, α3β1, and TGF-βR1 (Fig. 3, white) were seen mainly at the periphery of the epithelial islands. In comparison, cells expressing the nonpermissive H245A mutant α3 under the same conditions showed much less colocalization of the three receptors (Fig. 3, top). The mean white pixel intensity in multiple peripheral fields of six separate cellular islands for each cell type was ∼10 times higher on wt as compared with H245A integrin–expressing cells ($P < 0.001$). It is noteworthy that detection of TGF-βR1 (Fig. 3, green) in surface coclusters with α3β1 and E-cadherin was enhanced by a blockade of clathrin-dependent endocytosis and stimulation with TGF-β1. TGF-β1 is known to induce clathrin-mediated receptor turnover (Runyan et al., 2005), and the images in Fig. 3 were obtained in the presence of monodansylcadaverine (MDC), an inhibitor of this turnover pathway. Merged images without and with clathrin pathway blockers and TGF-β1 stimulation are provided in Fig. S4 A (available at http://www.jcb.org/cgi/content/full/jcb.200806067/DC1).

To allow for further analysis of E-cadherin–TGF-βR1 complexes, α3 wt epithelial or H245A mutant cells stably expressing a myc-tagged TGF-βR1 were established. E-cadherin was present in the myc–TGF-βR1 immunoprecipitates whether the integrin associates with E-cadherin or not (Fig. 4 A). Immunoprecipitation (IP) of myc–TGF-βR1 consistently coprecipitated wt α3 integrin but failed to coprecipitate the nonpermissive H245A mutant (Fig. 4 B), suggesting that the integrin likely associates with TGF-βR1 indirectly through E-cadherin. Additional experiments revealed that <10% of the total E-cadherin associated with TGF-βR1 by co-IP (unpublished data), raising the possibility that TGF-βR1 only associates with a small subfraction of cell surface E-cadherin.

The extent of E-cadherin–TGF-βR1 coclustering was not obviously different at baseline between α3 wt and H245A mutant cells. However, when clusters were visualized 24 h after TGF-β1 stimulation, coclusters of E-cadherin and TGF-βR1 disappeared from the surface of α3 wt cells, whereas these coclusters persisted on cells expressing the H245A mutant (Fig. 4 C and Fig. S4 B). Quantification of the total extent of yellow pixels as a fraction of the sum of both the yellow and red pixels in multiple random fields indicated that this fraction dramatically decreased in α3 wt cells (Fig. 4 D). The exact opposite was seen in H245A cells stimulated with TGF-β1, in which this fraction actually increases, reflecting persistence of surface E-cadherin–TGF-βR1 clusters concurrent with loss of overall surface E-cadherin. Overall turnover of TGF-βR1 induced by TGF-β1 was not impacted (Fig. 4 E), indicating that α3 specifically determines the fate of TGF-βR1–E-cadherin complexes but not all TGF-β receptor turnover pathways. The observation that E-cadherin and TGF-βR1 receptors colocalize and appear to internalize together in response to TGF-β1 raises the possibility that transcription factors known to be associated with these receptors, i.e., β-catenin and receptor-associated Smads, could also interact. We next investigated this possibility.

β-Catenin–p-Smad2 complex formation requires TGF-β1, α3 membrane complex, and endocytosis

As shown in Fig. 5 A, epithelial cells expressing wt α3 formed clear β-catenin–p-Smad2 complexes 1 h after TGF-β1 stimulation, whereas α3-null or H245A mutant cells failed to support formation of this transcriptional complex. The appearance of β-catenin–p-Smad2 complexes did not result in canonical β-catenin signaling as judged by the lack of response of the TOPFlash T cell factor/catenin reporter to TGF-β1 in transiently transfected α3 wt cells (unpublished data), which is consistent with prior evidence that TOPFlash is not activated directly by TGF-β1 stimulation in other cell types (Labbe et al., 2000). Also, we could not detect β-catenin in the nucleus by direct immunostaining at any time point after TGF-β1 stimulation. However, the catenin is functionally involved in TGF-β1 signaling, which is indicated by near-complete suppression of induction of α-SMA and PAI-1 protein in α3 wt cells stably transfected with a dominant-negative version of β-catenin (Fig. 5 B, left). To further test this point, α3-expressing cells were stably transfected with short hairpin RNA (shRNA) blocking β-catenin expression or a nonblocking shRNA, and the response to TGF-β1 was again assessed. Compared with control, knockdown of β-catenin expression by >50% markedly attenuated the α-SMA response to TGF-β1 without decreasing the p-Smad2 response (Fig. 5 B, right), confirming the importance of β-catenin in the cellular response to TGF-β1 signaling.

The formation of β-catenin–p-Smad complexes after TGF-β1 stimulation was found to require α3β1-dependent endocytosis because both clathrin inhibitors MDC and chlorpromazine inhibited β-catenin–p-Smad complex formation in α3 wt cells (Fig. S5 A, available at http://www.jcb.org/cgi/content/full/jcb.200806067/DC1). If β-catenin–p-Smad complexes are formed during or soon after receptor internalization, it is unclear how β-catenin would move from its physical interaction with E-cadherin to form transcriptionally active complexes with p-Smad2. An attractive possibility is tyrosine phosphorylation of β-catenin, especially tyrosine 654, which is known to lead to dissociation of β-catenin from E-cadherin (Piedra et al., 2001). Indeed, we found that Y-654 was phosphorylated within 60 min after active TGF-β1 addition to the cultures. This phosphorylation absolutely required the presence of a permissive α3β1.

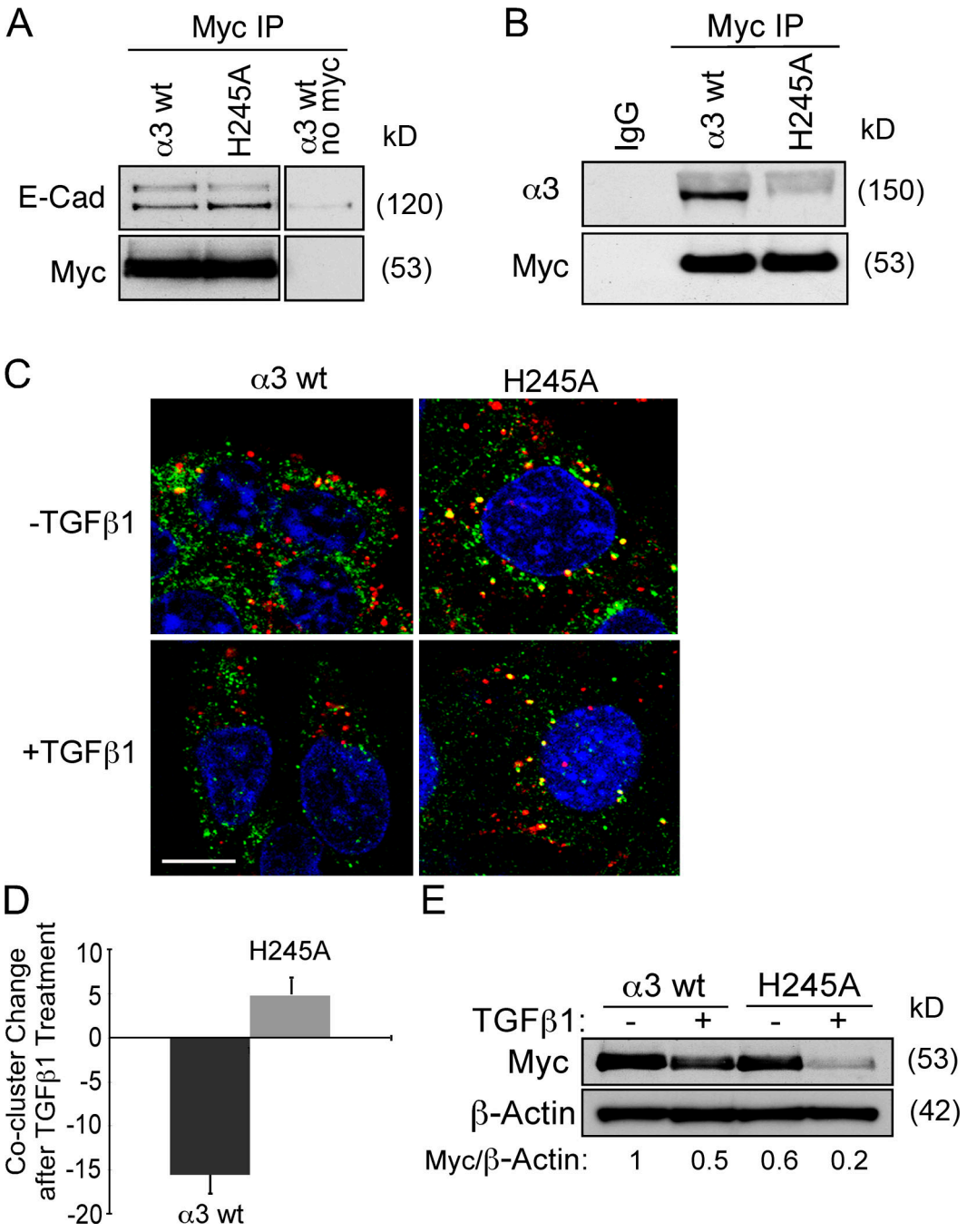

Figure 4. **Turnover of E-cadherin and TGF-βR1 complexes requires α3β1 integrin association.** (A) E-cadherin immunoprecipitates equally well with myc–TGF-βR1 in both α3 wt cells and H245A mutant cells. Myc IP from α3 wt cells without myc-TGF-βR1 expression (α3 wt no myc) was used as a negative control. (B) α3 integrin only immunoprecipitates with myc–TGF-βR1 in α3 wt cells but not in H249A mutant cells. (C) Confocal microscopy analysis of the effect of TGF-β1 on E-cadherin–TGF-βR1 coclusters in α3 wt and H245A mutant cells. After overnight treatment with TGF-β1, cell surface E-cadherin was clustered, fixed, and stained with anti-myc antibody. α3 wt cells show decreased E-cadherin–TGF-βR1 coclusters after TGF-β1 stimulation, whereas E-cadherin–TGF-βR1 coclusters persist in H245A mutant cells. Red, E-cadherin; green, TGF-βR1; blue, DAPI. (D) Quantification of E-cadherin–TGF-βR1 complex turnover. After TGF-β1 stimulation, the fraction of E-cadherin–TGF-βR1 coclusters decreases in α3 wt cells but increases in H245A mutant cells. (E) Immunoblotting of total myc–TGF-βR1 before and after TGF-β1 stimulation overnight showing that the overall turnover of TGF-βR1 is not different between α3 wt and H245A mutant cells. All of the aforementioned experiments have been performed at least three times with similar results. Error bars indicate standard deviation. Bar, 1 μm.

Neither α3-null cells nor cells bearing the H245A mutant phosphorylated β-catenin at Y-654 in response to TGF-β1 stimulation (Fig. 5 C).

To examine whether endocytosis is important for β-catenin tyrosine phosphorylation, α3 wt cells were serum starved, exposed to an inhibitor of clathrin-mediated endocytosis (MDC), and stimulated with TGF-β1. As shown in Fig. 5 D, pY654–β-catenin was absent in the inhibitor-treated cells, indicating that this phosphorylation requires endocytosis. Note that the blockade of endocytosis had no clear

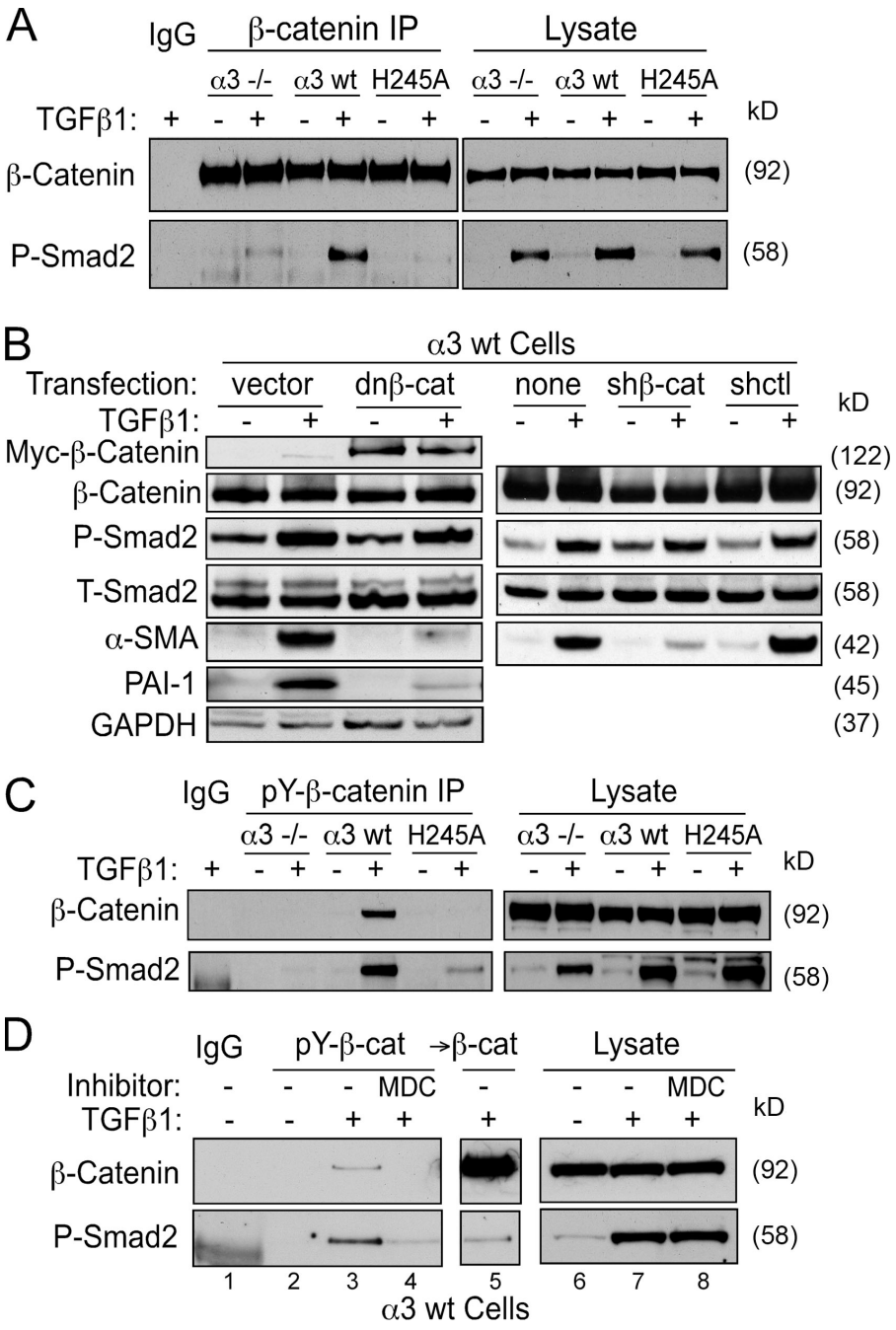

Figure 5. **β-Catenin–p-Smad2 complex formation requires wt α3 and is essential for TGF-β1–mediated up-regulation of mesenchymal genes.** (A) β-Catenin effectively immunoprecipitates p-Smad2 in α3 wt cells but not in $\alpha3^{-/-}$ or H245A mutant cells after TGF-β1 stimulation. (B) Inhibition of β-catenin by expression of a dominant-negative β-catenin (dnβ-cat; left) or an shRNA against β-catenin (shβ-cat; right) in α3 wt cells inhibits TGF-β1–induced α-SMA but not Smad2 phosphorylation compared with wt α3 cells transfected with vector alone, nonsilencing control shRNA (shctl), or left nontransfected. (C) Tyrosine phosphorylation of β-catenin and formation of the pY654–β-catenin–p-Smad2 complex is only detected in α3 wt cells treated with TGF-β1 but not in $\alpha3^{-/-}$ or H245A mutant cells. (D) Tyrosine phosphorylation of β-catenin (β-cat) and formation of the pY654–β-catenin–p-Smad2 complex in α3 wt cells requires endocytosis. α3 wt cells untreated or exposed to 300 μM of the clathrin inhibitor MDC were stimulated with TGF-β1 and analyzed for pY654–β-catenin–p-Smad2 complexes by co-IP. The supernatant from pY654–β-catenin IP was subsequently immunoprecipitated with total β-catenin antibody. Lane 3 shows p-Smad associated with pY654–β-catenin, and lane 5 shows the remainder of p-Smad2 on β-catenin after pY654–β-catenin depletion. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. All of the aforementioned experiments have been performed at least three times with similar results.

effect on overall levels of p-Smad2 (Fig. 5 D, right) but markedly attenuated formation of complexes as judged by co-IP. Interestingly, re-IP of total β-catenin in the supernatant after pY654–β-catenin IP resulted in little p-Smad2 (Fig. 5 D), and pY654–β-catenin mAb coimmunoprecipitated similar amounts of p-Smad2 as antibodies to total β-catenin (not depicted), indicating that p-Smad2 preferentially associates with tyrosine-phosphorylated β-catenin, and the principal form of β-catenin in β-catenin–Smad complexes is Y-654 phosphorylated. These data imply that the internalization of colocalized E-cadherin and TGF-βR1 (Fig. 4) allows β-catenin to be tyrosine phosphorylated, dissociate from E-cadherin, and form complexes with p-Smad2.

Catenin–Smad complex formation is regulated by cell contacts

The finding of α3β1-dependent β-catenin–p-Smad2 complexes raised the intriguing possibility that formation of such complexes is regulated at the cell surface by determinants of an assembly of complexes containing α3β1, E-cadherin, and TGF-βR1. Therefore, we asked whether limiting the availability of either α3β1 or E-cadherin would influence the formation of β-catenin–p-Smad2 complexes and the subsequent transcriptional responses. To vary access of E-cadherin to TGF-β1 receptors, we used two approaches. First, we varied the culture medium calcium concentration. Under high calcium conditions (~1.8 mM), α3 wt cells show a tight clustered phenotype (Fig. 6 A). Clustering

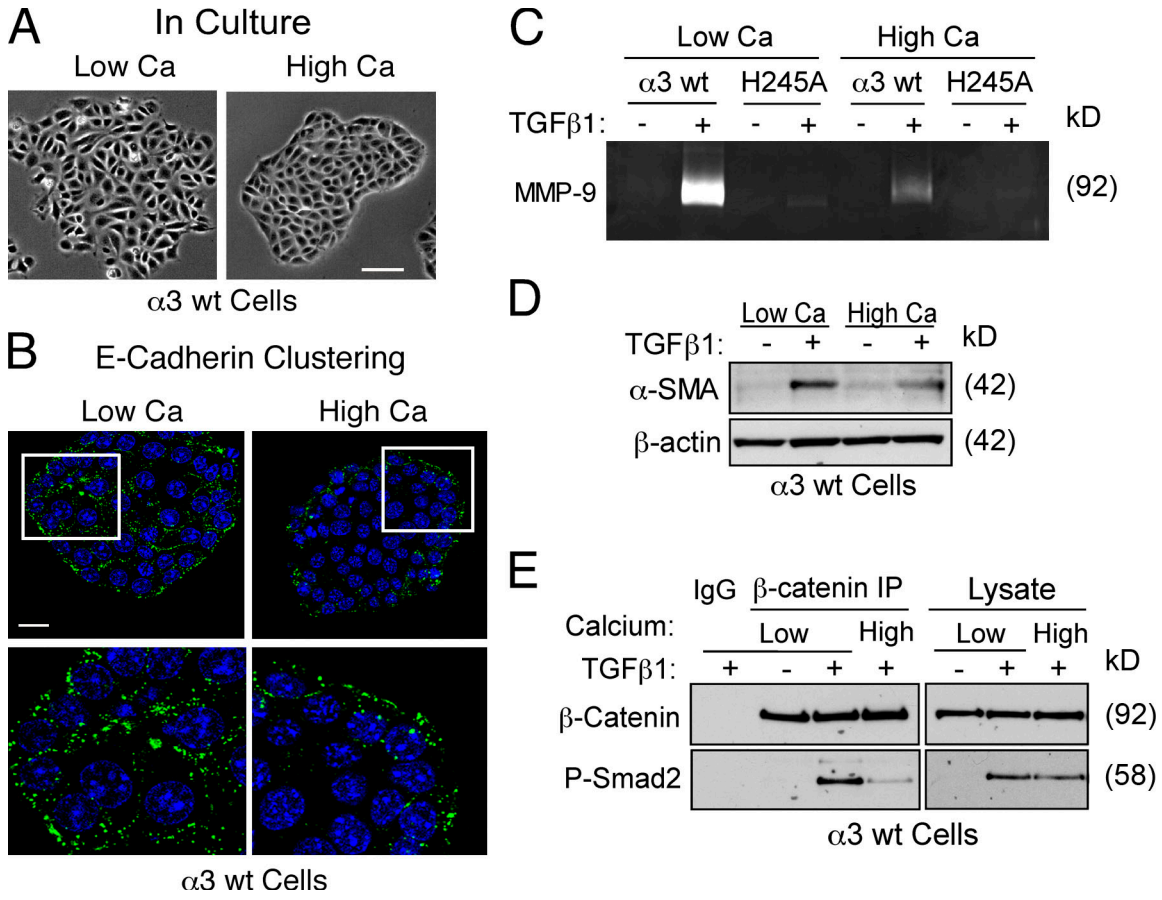

Figure 6. **Effect of calcium on TGF-β1–mediated signaling and β-catenin–p-Smad2 complex formation.** (A) Phase photographs of α3 wt cells cultured in either low (125 μM) or high (1.8 mM) calcium media showing cells cultured in high calcium media form tight cell–cell contact, whereas cells cultured in low calcium media have loose cell–cell contact. Bar, 50 μm. (B) Confocal microscopy of E-cadherin clusters in α3 wt cells cultured under either low (125 μM) or high (1.8 mM) calcium conditions. α3 wt cells cultured in high calcium media show that E-cadherin clusters occur mainly in cells at the periphery of the colony, whereas α3 wt cells cultured in low calcium media form E-cadherin clusters throughout the colony. The boxed areas are shown in higher magnifications in the bottom panels. Bar, 2 μm. (C) Serum-starved α3 wt and H245A mutant cells under high or low calcium conditions were stimulated with TGF-β1 for 48 h. Supernatants were removed from the plates and concentrated for zymography. α3 wt cells cultured in low calcium show higher MMP-9 levels than α3 wt cells cultured in high calcium after TGF-β1 stimulation. (D) Serum-starved α3 wt cells in high or low calcium media were stimulated with TGF-β1 for 48 h, and the lysates were blotted for α-SMA. α-SMA is only up-regulated in cells cultured in low calcium medium and not in high calcium condition. (E) Cells cultured under low calcium condition show increased β-catenin–p-Smad2 complex formation. α3 wt cells cultured in either high or low calcium medium were treated with TGF-β1, and the lysates were subjected to β-catenin IP followed by p-Smad2 immunoblotting. The β-catenin–p-Smad2 complex formation is only seen with cells cultured in low calcium condition and not in high calcium condition. All of the aforementioned experiments have been performed at least three times with similar results.

of E-cadherin on live cells is mainly confined to the periphery of well-compacted epithelial cell islands (Fig. 6 B), and there are relatively little β-catenin–p-Smad2 complexes formed after TGF-β1 stimulation (Fig. 6 E). In contrast, under low calcium conditions (~125 μM Ca^{2+}), there is more extensive E-cadherin clustering (Fig. 6 B) and complex formation (Fig. 6 E). Again, there is a direct correlation between the degree of β-catenin–p-Smad complexes and the induction of MMP-9 (Fig. 6 C) and α-SMA (Fig. 6 D) after TGF-β1 stimulation. Note that the complexes are observed within 60 min of exposure of the cells to TGF-β1, whereas the protein responses are measured after 48 h. In a second approach, we varied the plating density of the epithelial cells 24 h before TGF-β1 stimulation. At a high density in which there is extensive E-cadherin–dependent cell–cell contact, little or no β-catenin–p-Smad complexes were observed (Fig. S5 B) and little α-SMA was induced (not depicted). At low plating density, i.e., when E-cadherin is unengaged, both transcriptional complexes and induction of α-SMA were robust, confirming the critical role of the extent of adherens junction formation as a regulator of cellular responses to TGF-β1.

When α3 wt cells were cultured on Ln5, sequestering α3β1 to the basal surface, β-catenin–p-Smad2 complexes failed to form (Fig. 7 A), and the α-SMA transcriptional response to TGF-β1 was again attenuated (Fig. 7 C, left). In contrast, on purified Fn, complex formation and α-SMA induction is robust. To further test the role of Ln5 engagement in regulating TGF-β1 responses, we repeated the experiments using G163A mutant α3-expressing cells that are unable to engage Ln5, and thus, Ln5 would be predicted to have less influence on the TGF-β1 responses of these cells. Indeed, β-catenin–p-Smad2 complexes were readily observed when G163A cells were plated onto either Fn or Ln5 (Fig. 7 B), and the α-SMA induction was equivalent on either matrix (Fig. 7 C, right). Collectively, these data indicate that the α3β1–E-cadherin–TGF-βR1

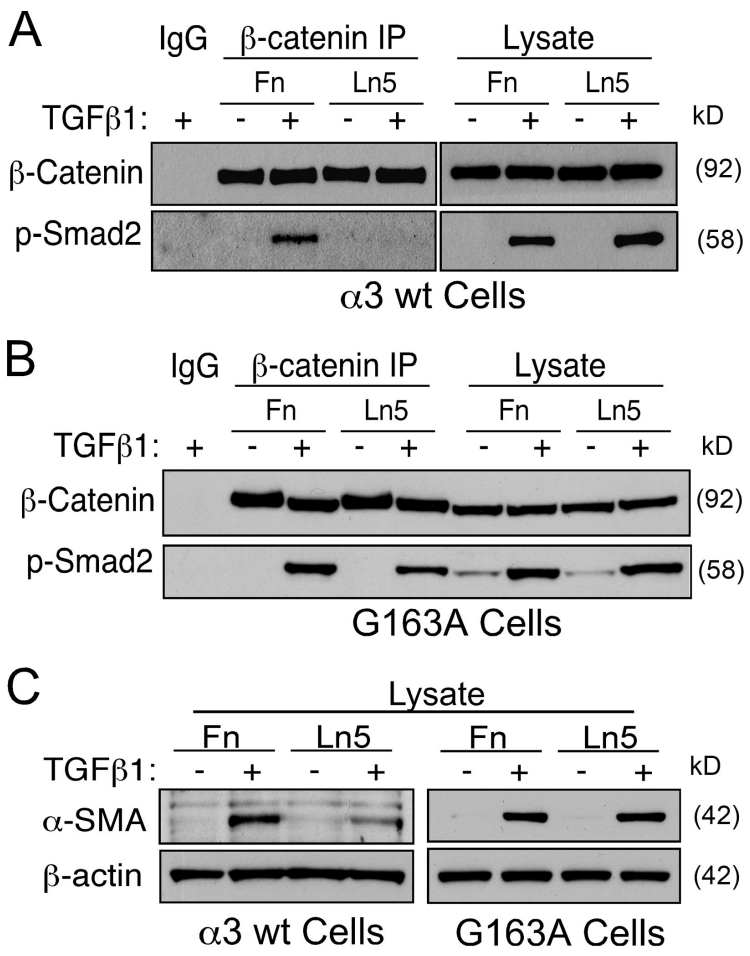

Figure 7. **Regulation of β-catenin–p-Smad2 complex formation by extracellular matrices.** (A) α3β1 engagement on Ln5 limits the formation of β-catenin–p-Smad2 complex formation. Lysates of TGF-β1–stimulated α3 wt cells from either Fn- or Ln5-coated plates were subject to β-catenin IP followed by p-Smad2 immunoblotting. The β-catenin–p-Smad2 complex formation is only seen with cells plated on Fn and not with Ln5. (B) Ln5 does not limit formation of β-catenin–p-Smad2 complexes in cells unable to engage Ln5 through α3β1 (G163A mutant cells). Lysates of TGF-β1–stimulated G163A mutant cells from either Fn- or Ln5-coated plates were subjected to β-catenin IP followed by p-Smad2 immunoblotting. The β-catenin–p-Smad2 complex formation is seen with cells plated on both Fn and Ln5. (C) α3β1 engagement on Ln5 suppresses TGF-β1–induced α-SMA up-regulation. Up-regulation of α-SMA is suppressed in α3 wt cells plated on Ln5 but not in G163A mutant cells on Ln5. The aforementioned experiments have been performed three times with similar results.

membrane complexes can coordinate cues from cell–matrix and cell–cell interactions to determine the cellular response to TGF-β1.

Discussion

The findings reported in this study identify a previously unrecognized role for the prominent epithelial integrin α3β1 in TGF-β1 signaling, providing evidence that the integrin functions as a sensor of cell contacts to regulate TGF-β1 signaling. The essential function of α3β1 appears to depend on the presence of surface complexes on epithelial cells, which are comprised of a subfraction of the surface pool of α3β1, E-cadherin, and TGF-βR1, which critically influence the signaling response to TGF-β1. The components of this complex by itself reveals the likely elements of its function, as modeled in Fig. 8. Surface complexes of E-cadherin and TGF-βR1 bring two key transcription factors involved in induction of EMT into spatial proximity: β-catenin and receptor Smads (Chilosi et al., 2003; Kalluri and Neilson, 2003; Yook et al., 2006). After TGF-β1 stimulation, the surface complexes are internalized, and β-catenin is phosphorylated on Y-654. The formation of stable complexes between pY654–β-catenin and p-Smad2 and the appearance of these complexes strongly correlate with subsequent EMT-related protein expression (Figs. 5–7). Both internalization and β-catenin tyrosine phosphorylation require the third component of the complex, α3β1. The unique function of α3β1 in this context appears to reside in its affinity for E-cadherin, without which E-cadherin–TGF-βR1 complex internalization is impaired, and this signaling system does not operate. Conceptually, these findings are similar to prior evidence in another system in which integrin engagement is found to regulate growth factor signaling at least in part by altering pathways of endocytosis that influence the quality and duration of receptor signaling (del Pozo et al., 2004). However, our findings also reveal a completely new point of intersection between β-catenin and Smad signaling pathways (Lei et al., 2004), and the linkage of this point of intersection to a dynamic interplay between adhesion receptors and their normal pericellular contacts provides new understanding for how the extracellular environment can regulate the epithelial cell response to TGF-β1 (Masszi et al., 2004; Thiery and Sleeman, 2006).

TGF-β1 has been previously reported to promote tyrosine phosphorylation of β-catenin (Tian and Phillips, 2002), although the specific site of phosphorylation and its functional significance in TGF-β1 signaling has been unknown. Our finding of integrin-dependent tyrosine phosphorylation of Y654–β-catenin is important because phosphorylation of β-catenin at Y654 is known to promote both dissociation of β-catenin from E-cadherin and stabilization of β-catenin from ubiquitination and degradation (Brembeck et al., 2006). Therefore, independently of Wnt signaling, our findings indicate that TGF-β1 can

promote a pathway of cross talk with β-catenin by generating stable pY654–β-catenin–Smad complexes. The data indicate that only a fraction of the β-catenin is phosphorylated, and presumably, this reflects, at least in part, the pool internalized with E-cadherin and TGF-βR1 after TGF-β1 stimulation. However, internalization alone does not appear to be sufficient, as α3-null cells, even though they display unstable surface E-cadherin, fail to phosphorylate β-catenin in response to TGF-β1. We are uncertain of the tyrosine kinases responsible for Y654 phosphorylation. At least three different tyrosine kinases have been reported to phosphorylate this site: c-src (and possibly other src family members; Roura et al., 1999), Bcr-Abl (Coluccia et al., 2007), and hepatocyte growth factor receptor Met (Zeng et al., 2006). In addition, the TGF-β1 receptor complex itself has recently been shown to have tyrosine kinase activity (Lee et al., 2007). As src family kinases are known to associate with the cytoplasmic tails of β1 integrins (Hynes, 2002), the requirement for the integrin likely involves a src family kinase. However, the exact kinase and how this kinase activity is locally regulated by α3β1 after TGF-β1 stimulation remains to be defined in future work.

A recent study described defective TGF-β1 signaling in α3β1-deficient murine keratinocytes and attributed the defect to higher levels of the inhibitory Smad, Smad7 (Reynolds et al., 2008). Corresponding to the higher Smad7 levels was lower overall levels of TGF-β1 receptors and lower Smad2/3 phosphorylation after TGF-β1 stimulation, which is consistent with the known effects of Smad7 on the TGF-β1 signaling pathway. However, neither in the kidney epithelial cell line studied here nor in α3β1-deficient primary lung epithelial cells were we able to detect altered levels of Smad7. Consistent with our data, overall levels of TGF-β1 receptors were not lower in α3-deficient cells, and Smad2/3 phosphorylation was not lower after TGF-β1 stimulation (Fig. 5). Together, these observations may imply that the impact of α3β1 on TGF-β1 signaling operates through different pathways in different cells but may also suggest that this integrin may critically influence TGF-β1 signaling in many, if not all, epithelial cells.

We have previously reported that stable overexpression of uPAR in kidney epithelial cells promotes an EMT response via interactions of uPAR with α3β1 (Zhang et al., 2003). The functionally important H245A α3 point mutation used in this study was discovered in a screen of several integrin β propeller mutants for inhibitors of uPAR-dependent EMT. Cells expressing the H245A mutant were found to have disrupted uPAR–α3β1 interactions and attenuated EMT compared with cells expressing wt α3. However, basal epithelial cells express little or no uPAR, and uPAR is a known TGF-β1 target gene (Yue et al., 2004). Therefore, we asked whether the response of these cells to TGF-β1 involved uPAR. Surprisingly, H245A-expressing cells were found to have little or no response to active TGF-β1, implying a critical role for α3β1 in the early response of these cells even before induction of uPAR and leading to the series of experiments reported in this study. It is likely that uPAR, once induced by TGF-β1, further promotes signaling amplifying EMT, as has been recently reported in other systems (Lester et al., 2007). However, findings reported in this study indicate

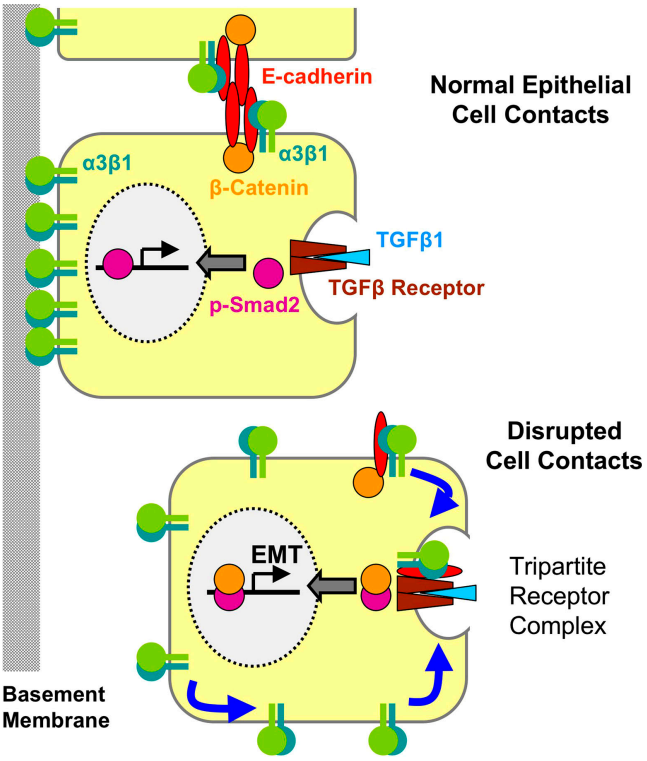

Figure 8. **Model of TGF-β1 signaling.** The schematic illustration summarizes the influence of α3β1 on TGF-β1 signaling as a function of cell–cell and cell–matrix contacts. For details see the Discussion.

that, independently of uPAR, α3β1 has a critical role in the initial TGF-β1 signaling leading to EMT.

One limitation of our observations is that it is unclear how pY654–β-catenin–p-Smad2 complexes operate to promote mesenchymal gene responses to TGF-β1. Although there is a strong correlation between formation of these complexes and initiation of EMT in both primary lung epithelial cells as well as the kidney epithelial cells primarily studied here, it is unclear what the critical promoter targets are for this complex. TGF-β1 does not strongly activate the canonical Lef1/T cell factor reporter, and activation of the canonical Smad-binding CAGA reporters is not different between cells expressing wt and H245A mutant α3β1 (Fig. S2), suggesting that a noncanonical signaling mechanism may be in play in this signaling pathway. Moreover, how can the pro-EMT signaling pathway involving p-Smad2 identified in this study be reconciled with recent findings that Smad2-null keratinocytes spontaneously develop EMT (Hoot et al., 2008), suggesting the possibility that p-Smad2 actually functions to suppress EMT? We note that total p-Smad2 levels after TGF-β1 stimulation are strongly and comparably induced in both responding α3 wt cells and nonresponsive α3-null and H245A mutant–expressing cells (Fig. 5). It is well known that cell-specific coactivators and repressors critically regulate the repertoire of responses to Smad signaling (Derynck and Zhang, 2003). This is consistent with the view that p-Smad2 may function to suppress EMT unless and until pY654–β-catenin–p-Smad2 complexes form under the conditions revealed by our experiments. If so, the set of TGF-β1 target genes

activated by pY654–β-catenin–p-Smad2 complexes and how this switches p-Smad2 from a suppressor to activator role in EMT are important areas for further investigation.

Materials and methods

Reagents

Recombinant active TGF-β1 was obtained from R&D Systems. Inhibitors PD98095 (MEK1), SB202190 (p38), SB431542 (TGF-β), JNK-II (SP600125), GSK3 inhibitor VII (TPK I inhibitor), and polyclonal p-Smad2 antibody were purchased from EMD. Rat anti–E-cadherin mAb was obtained from Invitrogen. E-cadherin pAb, mouse anti–E-cadherin mAb, and β-catenin mAb were purchased from BD. MDC, chlorpromazine hydrochloride, myc pAb, α-SMA mAb, and β-actin mAb were obtained from Sigma-Aldrich. Sheep anti–mouse PAI-1 pAb was obtained from American Diagnostica Inc. TGF-βRI pAb, β-catenin pAb, and myc mAb were purchased from Cell Signaling Technology. α3 pAb, pY654–β-catenin mAb, and fluorophore-labeled antibodies were purchased from Invitrogen. Secondary HRP-conjugated antibodies and Smad2/3 pAb were purchased from Santa Cruz Biotechnology, Inc. 804-G supernatant rich in Ln5 was provided by J.C. Jones (Northwestern University Medical School, Chicago, IL). Collagen I pAb was obtained from Abcam. Fn was obtained from Roche. Nuclear extraction kit and TOPFlash T cell factor reporter plasmid were obtained from Millipore.

Plasmid constructs and virus production

Myc–TGF-βR1 (provided by Y. Henis, Tel Aviv University, Tel Aviv, Israel; Gilboa et al., 1998) was cloned into a retroviral expression vector by M. Wheelock and K. Johnson's laboratory (University of Nebraska Medical Center, Omaha, NE). Dominant-negative β-catenin (provided by P. McCrea, University of Texas MD Anderson Cancer Center, Houston, TX) missing the C-terminal Lef-binding sites and fused with the transcriptional repressor *engrailed* was cloned into a retroviral expression vector (pWZL-blast). Retroviral supernatants were produced using Phoenix-E cells.

β-Catenin knockdown

Five different mouse β-catenin shRNAs in pLKO.1 lentiviral vector were obtained from Open Biosystems, and the lentiviral particles were packaged at the University of California, San Francisco core facility. Virus-infected α3 wt cells were selected with puromycin, and knockdown efficiency of β-catenin was screened. Cells stably expressing active shRNA (shβ-cat: 5′-CCGGCCCAAGCCTTAGTAAACATAACTCGAGTTATGTTTACTAAGGCTTGGGTTTTT-3′) and cells expressing inactive control shRNA (shctl: 5′-CCGGCCATTGTTTGTGCAGTTGCTTCTCGAGAAGCAACTGCACAAACAATGGTTTTT-3′) were compared for TGF-β1 response.

α3-Null kidney epithelial cell culture

α3-Null B12 cells expressing the wt α3 or point mutants (H245A and G163A) were cultured in DME supplemented with 10% FBS, 50 µg/ml zeocin, and penicillin-streptomycin as previously described (Zhang et al., 2003). All cells were sorted periodically to maintain surface levels of α3 comparable with wt. Unless otherwise indicated, cells were seeded in DME 10% FBS for 24 h and switched to F-12 serum-free media (~200 µM Ca^{2+}) with 4 ng/ml TGF-β1 for different time periods. Cells were analyzed by immunoblotting, IP, and Taqman real-time PCR as previously described (Zhang et al., 2003; Kim et al., 2006). For inhibitor experiments, the following concentrations were used: 100 nM JNK-II inhibitor, 20 µM SB202190, 10 µM PD98059, and 50 µM TPK I inhibitor.

IP and Western blotting

For co-IP of E-cadherin and α3 integrin, 1% Triton X-100 lysates were immunoprecipitated with E-cadherin mAb, and the immunoprecipitates were blotted for α3 integrin and E-cadherin. For co-IP of myc–TGF-βR1 and α3 integrin or E-cadherin, 1% NP-40 lysates were immunoprecipitated with myc mAb for α3 integrin co-IP or myc pAb for E-cadherin co-IP. The immunoprecipitates were blotted with α3 integrin pAb or E-cadherin mAb. For co-IP of β-catenin and p-Smad2, serum-starved cells were treated with 2 ng/ml TGF-β1 for 60 min, the RIPA (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, and 0.1% SDS) lysates were immunoprecipitated with β-catenin mAb or pY654–β-catenin mAb, and the immunoprecipitates were blotted with p-Smad2 pAb and β-catenin pAb. In some cases, cells were pretreated with clathrin inhibitors, incubated in high Ca^{2+}-containing medium, or seeded on Fn or Ln5 surfaces before TGF-β1 treatment and co-IP.

Time-lapse microscopy and bright field microscopy of α3 wt and H245A mutant cells

Serum-starved cells in 60-mm dishes were treated with 4 ng/ml TGF-β1 and maintained in a mini incubator (5% CO_2 at 37°C) under a microscope (TE300; Nikon), and time-lapse microscopy (10× magnification) was performed for 48 h (1 frame/10 min) using a digital imaging system and the Simple PCI software (Hamamatsu Photonics). Cells were filmed over the same times after plating to eliminate nonspecific differences. Bright field images of cells in culture were taken under a microscope (TE300; 20× magnification) using a camera (Spot; Diagnostic Instruments, Inc.) and processed using Photoshop (Adobe).

Immunofluorescence, coclustering, and confocal microscopy

Cells were seeded in chamber slides and cultured for 2–3 d until 50% confluent. For E-cadherin staining, cells treated with TGF-β1 for 48 h were incubated with rat anti–E-cadherin antibody followed by secondary anti–rat IgG–Alexa Fluor 568 antibody. For coclustering, cells were first incubated with rat anti–E-cadherin or mouse anti-α3 antibodies on ice for 30 min and washed with PBS. E-cadherin or α3 was clustered at 37°C with Alexa Fluor 568–conjugated anti–rat or Alexa Fluor 350–conjugated anti–mouse secondary antibodies for 60 min. Washed cells were fixed in methanol, blocked with 10% goat serum PBS, and stained with anti-myc or anti–TGF-βR1 primary antibodies and Alexa Fluor 488–conjugated anti–rabbit secondary antibody. IgG isotypes were used as negative controls. Confocal microscopy was performed on a laser-scanning microscope (LSM 510; Carl Zeiss, Inc.). Images were captured with a 63× oil immersion objective, analyzed with the Image Browser software (LSM; Carl Zeiss, Inc.), and processed with Photoshop. For quantitative analysis of E-cadherin–TGF-βR1 coclusters, 16 random confocal microscopy fields were used for each treatment (α3 wt and H245A mutant cells ± TGF-β1 treatment). Data are expressed as the fractional difference (±SD) between E-cadherin–TGF-βR1 coclusters (total yellow pixels/total yellow + red pixels) in cells stimulated with TGF-β1 minus that of unstimulated cells for both α3 wt and H245A mutant cells. For quantitative analysis of E-cadherin–α3β1–TGF-βR1 coclusters, the exported confocal images were converted to black and white, and the fraction of white in each of at least six random fields along the edges of each of six colonies for each cell line (α3 wt and H245A mutant cells) was measured using the ImageJ software (National Institutes of Health). Statistical analysis was conducted using a Student's two-tailed *t* test for independent groups.

Gelatin zymography

Supernatants from cells cultured in F-12 + 0.001% BSA treated with TGF-β1 were collected and concentrated 10-fold. Protein concentration was normalized on cell number and volume. Lysates were prepared in 3× Laemmli SDS sample buffer, and 10% gelatin PAGE was performed (Invitrogen) and analyzed for MMP activity as described previously (Wei et al., 2007).

Mouse type II cell isolation and treatment with Cre recombinase

Isolation of type II alveolar cells from α3 conditional knockout mice was performed as described previously (Corti et al., 1996) with recent modifications (Kim et al., 2006). Purified primary type II cells were cultured on Matrigel/collagen type I/small airway growth medium (70:5:25 vol/vol) as described previously (Rice and Leinwand, 2003). Cells were mixed with adeno-Cre virus (provided by L. Wu, University of California, Los Angeles, Los Angeles, CA) or adeno-GFP virus at 10–20 plaque-forming U/cell before seeding cells on Matrigel/collagen and were incubated with virus for 96 h. Cells were removed from the Matrigel/collagen and plated on Fn to activate endogenous TGF-β1 signaling (Kim et al., 2006).

Online supplemental material

Videos 1 and 2 show TGF-β1–induced cell motility and morphological changes in α3 wt cells (Video 1) and H245A mutant cells (Video 2). Fig. S1 shows TGF-β1–induced expression of MMP-9, PAI-1, and α-SMA in α3 wt but not in H245A mutant cells. Equivalent Smad-dependent signaling in α3 wt, H245A, and G163A mutant cells are shown in Fig. S2. Fig. S3 shows that inhibition of JNK MAPK blocks TGF-β1–induced phenotype change but not expression of MMP-9 and α-SMA. Colocalization and turnover of α3β1, E-cadherin, and TGF-βR1 on cell surfaces of α3 wt cells are identified in Fig. S4. Fig. S5 presents regulation of TGF-β1–mediated β-catenin–p-Smad2 complex formation. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200806067/DC1.

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