Crosstalk of the mTOR network with stress granules and the TGF-beta pathway
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CHAPTER 6

**TSC1 Activates TGF-b-Smad2 Signaling in Growth Arrest and Epithelial-to-Mesenchymal Transition**

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SUMMARY
The tuberous sclerosis proteins TSC1 and TSC2 are key integrators of growth factor signaling. They suppress cell growth and proliferation by acting in a heteromeric complex to inhibit the mammalian target of rapamycin complex 1 (mTORC1). In this study, we identify TSC1 as a component of the transforming growth factor β (TGF-β)-Smad2/3 pathway. Here, TSC1 functions independently of TSC2. TSC1 interacts with the TGF-β receptor complex and Smad2/3 and is required for their association with one another. TSC1 regulates TGF-β-induced Smad2/3 phosphorylation and target gene expression and controls TGF-β-induced growth arrest and epithelial-to-mesenchymal transition (EMT). Hyperactive Akt specifically activates TSC1-dependent cytostatic Smad signaling to induce growth arrest. Thus, TSC1 couples Akt activity to TGF-β-Smad2/3 signaling. This has implications for cancer treatments targeting phosphoinositide 3-kinases and Akt because they may impair tumor-suppressive cytostatic TGF-β signaling by inhibiting Akt- and TSC1-dependent Smad activation.

Highlights
• TSC1 is required for TβR-I-Smad2/3 association and Smad2/3 phosphorylation
• TSC1 regulates Smad2/3 phosphorylation independently of TSC2 and Rheb/ mTORC1
• TSC1 is required for TGF-β1-induced growth arrest and EMT
• TSC1 positively couples insulin-Akt signaling to the TGF-β-Smad2/3 pathway
INTRODUCTION
Transforming growth factor β (TGF-β) signaling has emerged as a major regulator of development, tissue homeostasis, and disease. Mutations in TGF-β pathway components cause developmental defects, and perturbations in TGF-β signaling in the adult are linked to tumorigenesis and metastasis (Meulmeester and Ten Dijke, 2011). The TGF-β ligand signals through a hetero-tetrameric TGF-β receptor type I (TβR-I)-TGF-β receptor type II (TβR-II) complex that phosphorylates the Smad2 and Smad3 proteins within their C-terminal SSXS serine motifs. Phospho-activated Smad2/3 form a complex with Smad4 that accumulates in the nucleus and regulates numerous target genes. Depending on the cellular context, these transcriptional events induce growth arrest and apoptosis, but also EMT, cell migration and invasiveness (Massagué, 2012). The versatility of TGF-β signaling underlies its multifaceted role in cancer, frequently switching from tumor-suppressive to tumor-promoting functions in the course of tumor development (Tian and Schiemann, 2009).

Increasing evidence suggests that both cytostatic and pro-metastatic actions of canonical TGF-β-Smad2/3 signaling are modulated by components of the phosphoinositide 3-kinase (PI3K), Akt, and mammalian target of rapamycin complex 1 (mTORC1) pathway, thus linking mitogenic growth factor signaling to the TGF-β pathway (Bakin et al., 2000, Birchenall-Roberts et al., 2004, Conery et al., 2004, Remy et al., 2004, Song et al., 2006, Xue et al., 2012, Zhang et al., 2012). The tuberous sclerosis protein (TSC) TSC1-TSC2 complex is negatively regulated by insulin/IGF-1-PI3K-Akt signaling. TSC1-TSC2 suppresses the activity of the mTORC1 multiprotein complex (comprising mTOR kinase and the core scaffold protein Raptor) through inhibition of the mTORC1 activator Ras homolog enriched in brain (Rheb) (Orlova and Crino, 2010). Active mTORC1 phosphorylates its targets ribosomal protein S6 kinase (p70-S6K) and eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) to control cellular growth and proliferation (Laplante and Sabatini, 2012, Shimobayashi and Hall, 2014). Of note, mTORC1 and phospho-activated p70-S6K induce a negative feedback loop (NFL), which inhibits upstream PI3K/Akt activity (Hsu et al., 2011, Sengupta et al., 2010, Yu et al., 2011). In humans, disintegration of the TSC1-TSC2 complex, by mutations in either the TSC1 or TSC2 gene, leads to tuberous sclerosis complex, a multiorgan disease of benign tumors (Inoki and Guan, 2009). A function of TSC1-TSC2 in linking the insulin- and TGF-β pathways has so far not been addressed, leaving the role of the TSCs in TGF-β signaling ill-defined.
RESULTS

TSC1 Is Required for TGF-β1 Induced Smad2 /Phosphorylation, Nuclear Localization, and Target Gene Expression

To evaluate the roles of TSC1 and TSC2 in TGF-β signaling, we individually knocked down either TSC1 or TSC2 in HeLa cells by inducible shRNA expression (TSC1-i, TSC2-i). Expectedly, activation of the TGF-β-Smad2/3 pathway by TGF-β1 treatment resulted in phosphorylation of the SSXS motifs of Smad2 (S465/467) and Smad3 (S423/425) (pSmad2 and pSmad3) in control knockdown (Ctrl-i) cells (Figure 1A). Knockdown of either TSC1 or TSC2 strongly induced phosphorylation of p70-S6K at T389, due to derepression of mTORC1. Surprisingly, we observed that TSC1 but not TSC2 deficiency severely impaired Smad2 and Smad3 phosphorylation in response to TGF-β1 stimulation (Figure 1A). The ratio of pSmad2/total Smad2 and pSmad3/total Smad3 was significantly reduced by TSC1 deficiency, whereas it remained unchanged by TSC2 knockdown (Figure 1B). The pSmad inhibition observed in the TSC1 knockdown cell line TSC1-i #1 upon short-term TGF-β1 treatment (Figure 1A) was confirmed by a second shRNA sequence targeting TSC1 in a different exon (TSC1-i #2, Figure 1C). Also, after long-term TGF-β1 stimulation (24 and 48 hr), TSC1 knockdown by both sequences, TSC1-i #1 or TSC1-i #2, inhibited Smad phosphorylation (Figure S1A). Moreover, expression of a mutagenized TSC1-myc construct (TSC1-myc_non-targeted), which is not targeted by TSC1-i #1 knockdown (Figure S1B), rescued Smad2/3 phosphorylation in TSC1-i #1 knockdown cells (Figure S1C), further validating that TSC1 is required for Smad2/3 phosphorylation. Targeting TSC2 with an independent second shRNA sequence (TSC2-i #2, Figure 1D) did not show any effect on Smad phosphorylation. Consistent with these results obtained in HeLa epithelial cells, TSC1, but not TSC2, deficiency mitigated TGF-β1-induced Smad2/3 phosphorylation also in HEK293T cells (Figure S1D), indicating that TSC1 is a crucial component of TGF-β signaling in different cellular contexts. Notably, we found that BMP4 triggered phosphorylation of Smad1/5/8 was not affected by TSC1 deficiency (Figure 1E), suggesting that TSC1 specifically regulates Smad2/3 signaling. Previous studies have shown that TGF-β activates mTORC1 and p70-S6K via the PI3K-Akt-TSC1/2 axis in a cell-type-specific manner (Das et al., 2008, Kato et al., 2009, Lamouille and Derynck, 2007, Rahimi et al., 2009, Wu and Derynck, 2009) (see Figure S1D; please note that TGF-β1 does not induce p70-S6K-pT389 in epithelial HeLa cells, Figure 1A). Our results suggest that TSC1 is not only involved in TGF-β-dependent mTORC1 regulation, but also affects TGF-β-Smad signaling itself.
In keeping with the reduced Smad2/3 phosphorylation, nuclear Smad2/3 accumulation upon TGF-β1 stimulation was also decreased in HeLa TSC1-i cells, but not in TSC2-i cells (Figures 1F and 1G). This further corroborates a regulatory function of TSC1 toward Smad2/3. TGF-β-Smad signaling causes growth arrest in epithelial, neural, and hematopoietic cells by repressing mitogenic and inducing cytostatic target genes (Heldin et al., 2009), including the cyclin-dependent kinase inhibitors p21 and p57\(^{Kip2}\) (Lee et al., 2004, Lee et al., 2007, Nishimori et al., 2001, Pardali et al., 2005, Scandura et al., 2004). We found that TSC1, but not TSC2, knockdown suppressed the TGF-β1-dependent increase in p21 (Figure 1H) and p57\(^{Kip2}\) levels (Figures S1E and S1F). Thus, TSC1 positively controls Smad2/3 phosphorylation and nuclear translocation, and cytostatic TGF-β target gene expression.

**TSC1 Regulates Smad2/3 Phosphorylation Independently of the TSC1 TSC2 Effectors Rheb and mTORC1**

Because TSC1 deficiency, via suppression of TSC1-TSC2 complex activity, stimulates Rheb and mTORC1, we tested whether mTORC1 activity contributes to the regulation of Smad2/3 phosphorylation by TSC1. Inhibition of mTORC1 by rapamycin or PP242 efficiently suppressed aberrant mTORC1 activation in TSC1 knockdown cells as monitored by p70-S6K phosphorylation (Figure 2A). Yet, mTORC1 inhibition did not restore deficient Smad2/3 phosphorylation (Figures 2A and 2B). Also, several approaches to inhibit mTORC1 activity (rapamycin, PP242, shRNA targeting Raptor; Figure 2C), or Rheb (Figures 2D and 2E) in TSC1-positive cells did not affect TGF-β1-induced Smad2/3 phosphorylation. Thus, TSC1 regulates Smad2/3 phosphorylation independently of the canonical TSC1-TSC2 effectors Rheb and mTORC1.

**TSC1 Interacts with TβR-I/II and Smad2/3 and Required for TGF-β1 Induced TβR-I-Smad2/3 Association**

To explore the mechanisms via which TSC1 regulates Smad2/3 phosphorylation, we tested whether TSC1 knockdown reduces TGF-β receptor protein levels or alters levels of major TGF-β pathway modulators. TSC1 knockdown did not downregulate TβR-I or TβR-II levels (Figure S2A). Furthermore, TSC1 deficiency did not result in reduced levels of the positive modulator Smad anchor for receptor activation (SARA), which facilitates Smad phosphorylation, or increased levels of the inhibitory modulator Smad7 (Figure S2A).

We next hypothesized that TSC1 may modulate Smad2/3 phosphorylation by associating with Smad2/3 or the TGF-β receptor proteins. We assessed endog-
TSC1 Activates TGF-b-Smad2/3 Signaling

Figure 1 TSC1 Regulates TGF-β-Smad2/3 Signaling

(A) TSC1, but not TSC2, is required for TGF-β1-induced phosphorylation of Smad2/3. Inducible HeLa shRNA cell lines were treated with doxycycline to induce shRNA expression, or left untreated. Cells were starved and stimulated with TGF-β1 (5 ng/ml; this concentration was used in all experiments if not indicated otherwise) for 45 min as indicated. Please note that the Smad3-pS423/425 antibody occasionally detected a double band. For these cases, the signal corresponding to the molecular weight of Smad3 of 48 kDa (UniProt Consortium (2014), P84022-1) is marked by an arrow.

(B) Quantitation of three independent experiments as performed in (A); pSmad/total Smad in HeLa shRNA cells; one-way ANOVA, mean values ± SEM; **p < 0.01; *p < 0.05.

(C) Reduced Smad2/3 phosphorylation by independent second TSC1-i shRNA sequence (TSC1-i #2) upon TGF-β1 treatment. HeLa shRNA cells were starved and stimulated with TGF-β1 as indicated.

(D) Second independent knockdown of TSC2 (TSC2-i #2) does not affect TGF-β1-induced Smad2/3 phosphorylation. HeLa shRNA cells were starved and stimulated with TGF-β1 as indicated.

(E) TSC1 deficiency does not affect BMP4 signaling. HeLa shRNA cells were starved and stimulated with BMP4 for 30 min as indicated.

(F) Nuclear enrichment of Smad2/3 is impaired under TSC1 deficiency. Confocal immunofluorescence (IF) microscopy images of HeLa shRNA cells. Cells were starved, pretreated with the TβR-I inhibitor SB431542 (10 μM; 1 hr prior to TGF-β1), and stimulated with TGF-β1 (1 hr) as indicated. IF stainings with Smad2/3 antibody. Insert: A detailed view of an individual cell. Scale bar represents 10 μm.

(G) Quantitation of three independent experiments as performed in (F); percentage of cells with predominantly nuclear staining of Smad2/3; shown are mean values from three independent experiments with at least five independent fields of view per experimental condition of each experiment; one-way ANOVA, mean values ± SD; ***p < 0.001.

(H) TSC1, but not TSC2, is required for TGF-β1-induced expression of p21. HeLa shRNA cells were stimulated with TGF-β1 for 48 hr as indicated.

Immunoblot (IB) analysis of cell lysates (A, C, D, E, and H). ns, not statistically significant. See also Figure S1.

Enous TSC1-Smad2/3 association by proximity ligation assay (PLA), which due to its high sensitivity is a valuable tool to monitor transient and dynamic protein associations in situ (Söderberg et al., 2008), and found that TSC1 associates with Smad2/3 upon TGF-β1 treatment (Figures 3A and 3B; negative controls in Figure 3C). To test if TSC1 forms physical complexes with the Smad proteins, we performed co-immunoprecipitations. Indeed, TSC1-myc co-precipitated with both Flag-Smad2 and Flag-Smad3 (Figure S2B, negative control in Figure S2C). Mapping experiments with TSC1 truncation constructs (Hoogeveen-Westerveld et al., 2010) revealed that TSC1-Smad3 binding requires the N-terminal part of TSC1 (Figure S2D), which contains a putative transmembrane domain (amino acids 127–144) (van Slegtenhorst et al., 1997). Interestingly, TSC1-Smad3 interaction did not require the C-terminal coiled-coiled domain of TSC1 (amino acids 721–997) (UniProt Consortium, 2014; http://www.uniprot.org/uniprot/
Figure 2  TSC1 Dependent Phospho-Smad2 Regulation Does Not Involve mTORC1

(A) Impaired Smad activation by TGF-β1 in TSC1 knockdown is not restored by mTORC1 inhibition. HeLa shRNA cells were starved and pretreated with the mTOR inhibitors PP242 (200 nM) or rapamycin (200 nM) for 1 hr prior to TGF-β1 stimulation (45 min) as indicated.

(B) Quantitation of three independent experiments as performed in (A); pSmad/total Smad in HeLa shRNA cells; one-way ANOVA, mean values ± SEM.

(C) HeLa shRNA cells were cultivated in full medium and pretreated with PP242 (200 nM) or rapamycin (200 nM) for 1 hr prior to TGF-β1 stimulation as indicated.

(D) HeLa Rheb-i #1 and Rheb-i #2 shRNA cells were cultivated in full medium and stimulated with TGF-β1 as indicated.

(E) Quantitation of three independent experiments as performed in (D); pSmad/total Smad in HeLa shRNA cells, 45 min TGF-β1; one-way ANOVA, mean values ± SEM.

IB analysis of cell lysates (A, C, and D). ns, not statistically significant.
Figure 3 TSC1 Mediates TGF-β1 Induced TβR-I-Smad2/3 Association

(A and B) Analysis of endogenous TSC1-Smad2/3 association by proximity ligation assay (PLA). HeLa shRNA cells were starved and stimulated with TGF-β1 for 10 min as indicated. Confocal images (A) and quantitation (B) of PLAs. Red dots represent protein interaction events; DAPI nuclear staining in blue (this applies also for the following PLA data). (B) Area of interaction in square pixels. One-way ANOVA, mean values ± SD; ***p < 0.001; ns = not statistically significant.

(C) Negative control of PLA experiment depicted in (A). Confocal images of PLA performed with single primary antibodies, followed by incubation with both oligo-linked secondary antibodies (this applies also for the following PLA negative controls).

(D) TSC1, but not TSC2, interacts with TβR-I. Immunoprecipitation (IP) from lysates of Flag-TSC1 or Flag-TSC2 transfected HeLa cells was performed using either TβR-I-specific or control rabbit IgG antibodies (mock).

(E) TSC1 is required for endogenous TβR-I-Smad2/3 association in response to TGF-β1. HeLa shRNA cells were starved and stimulated for 2 to 20 min with TGF-β1 as indicated. Confocal images of PLAs.

(F) Negative control of PLA experiment depicted in (E). Scale bar (A, C, E, and F) represents 10 μm.

See also Figure S2.
TSC1 Activates TGF-β-Smad2/3 Signaling

Figure 4 TSC1 Links Insulin-Akt to TGF-β-Smad2/3 Signaling

(A and B) Constitutively active myristoylated Akt (myr-Akt) enhances TSC1-TβR-I association. HeLa cells were transfected with myr-HA-Akt or empty vector and stimulated with TGF-β1 for 12.5 min. Confocal images (A), and quantitation (B) of PLAs. Two tailed t test, mean values ± SD; ***p < 0.001.

(C) Negative controls of PLA experiment depicted in (A).

(D and E) myr-Akt enhances TβR-I-Smad2/3 association. Transfection and treatment as performed in (A). Confocal images (D) and quantitation (E) of PLAs. Two tailed t test, mean values ± SD; *p < 0.05.

(F) Negative controls of PLA experiment depicted in (D).

(G and H) myr-Akt stimulates Smad2/3 phosphorylation (G) and this is TSC1-dependent (H). HeLa cells were transfected with myr-HA-Akt or empty vector (G and H). Cells were cultivated in full medium (G) or starved and TGF-β1 (15 min) stimulated (H).

(I) HeLa shRNA cells were transfected with myr-HA-Akt or empty vector, starved, and stimulated with TGF-β1 (10 min) as indicated.

(J) myr-HA-Akt or empty vector transfected HeLa cells were treated with PP242 (200 nM) or rapamycin (200 nM) 2 hr prior to lysis as indicated.

(K and L) Insulin enhances TGF-β1-induced Smad2/3 phosphorylation (K) and this is TSC1-dependent (L). HeLa cells were starved and stimulated with insulin (100 nM, 2 hr) prior to TGF-β1 stimulation (15 min) as indicated.

Scale bar (A, C, D, and F) represents 10 μm. IB analysis of cell lysates (G–L).

See also Figure S3.

Q92574) (Figure S2D), which is involved in mediating TSC1-TSC2 interaction (Hoogeveen-Westerveld et al., 2010). This indicates that distinct TSC1 domains mediate TSC1-Smad and TSC1-TSC2 interactions, respectively. Furthermore, TSC1 co-precipitated with both TβR-I (Figure 3D; PLA of TSC1-TβR-I association in Figure S2E) and TβR-II (Figure S2F). TSC2 did not interact with TβR-I (Figure 3D) and TSC1 remained bound to TβR-I in TSC2 knockdown cells (Figure S2G), further supporting a TSC2-independent role of TSC1 in the TGF-β pathway.

TSC1’s interaction with TβR-I and Smad2/3 suggested that TSC1 regulates TβR-I-Smad2/3 association. We assessed this possibility by PLA. TGF-β1 treatment induced a strong and transient association of endogenous TβR-I-Smad2/3 in Ctrl-i cells at approximately 10 min of TGF-β1 stimulation (Figure 3E; negative controls in Figure 3F). TSC1 deficiency impaired TGF-β1-induced TβR-I-Smad2/3 binding, revealing a role for TSC1 in mediating the association of TβR-I with its substrates (Figure 3E). Interestingly, we observed TβR-I-Smad2/3 association also at later time points of TGF-β1 stimulation (Figure S2H; 40 min TGF-β1), indicating that multiple peaks of TSC1-dependent TβR-I-Smad2/3 association contribute to Smad2/3 phosphorylation.
TSC1 Activates TGF-β-Smad2/3 Signaling

We next tested if insulin/IGF-1-PI3K-Akt signaling, which regulates TSC1-TSC2 activity toward mTORC1, also affects TSC1’s function in the TGF-β pathway. Indeed, constitutively active myristoylated Akt (myr-Akt) strongly promoted TSC1-TβR-I (Figures 4A and 4B; negative control in Figure 4C), and TβR-I-Smad2/3 association (Figures 4D and 4E; negative control in Figure 4F). As previously reported, myr-Akt reduced TSC2 protein levels (Figure S3A) (Dan et al., 2002, Plas and Thompson, 2003) and decreased TSC1-TSC2 interaction (Figure S3B) (Inoki et al., 2002, Potter et al., 2002), confirming the functionality of myr-Akt in TSC1-TSC2 complex regulation. In line with its stimulating effect on TβR-I-Smad2/3 association (Figures 4D and 4E), myr-Akt expression enhanced Smad2/3 phosphorylation in a dose-dependent manner (Figure 4G). Absence of TGF-β1 in starved cells or treatment with the TβR-I inhibitor SB431542 prior to TGF-β1 stimulation prevented efficient enhancement of Smad phosphorylation by myr-Akt (Figure S3C). Hence, myr-Akt-induced Smad phosphorylation requires TβR-I activity. myr-Akt-induced Smad phosphorylation was inhibited by TSC1 deficiency (Figures 4H and S3D), but not by...
TSC1 Activates TGF-b-Smad2/3 Signaling

**Figure 6 TSC1 Is Required for TGF-β1 Dependent Growth Arrest and EMT in NMuMG Cells**

(A) TSC1 deficiency impairs TGF-β1-induced growth arrest in NMuMG cells. NMuMG shRNA cells were treated with TGF-β1 (24 hr) and subjected to propidium iodide staining followed by flow cytometry. Quantitation of four independent experiments with each condition being performed in duplicates or triplicates in each experiment. Results are shown as box plots representing median, 25th and 75th percentiles as boxes, and the range of data as bars; one-way ANOVA, **p < 0.01.

(B) TSC1 deficiency impairs Smad2/3 phosphorylation in NMuMG cells. Quantitation of three independent experiments showing pSmad/total Smad. NMuMG shRNA cells were starved and stimulated with TGF-β1 for 30 min; one-way ANOVA, mean values ± SEM; **p < 0.01; *p < 0.05.

(C) myr-HA-Akt or empty vector expressing NMuMG cells were stimulated with TGF-β1 for 15 min as indicated.

(D) myr-Akt induces growth arrest via Smad signaling. Propidium iodide staining as performed in (A) using stably myr-HA-Akt/empty vector expressing NMuMG shRNA cells. Box plot presentation as in (A); one-way ANOVA, **p < 0.01.

(E) TSC1 is required for expression of mesenchymal marker proteins. NMuMG shRNA cells were subjected to TGF-β1 treatment (72 hr) as indicated.

(F) Wide field fluorescence images of NMuMG shRNA cells, ± TGF-β1 (72 hr). Scale bar represents 30 μm.

(G) Stably myr-HA-Akt/empty vector expressing NMuMG Ctrl-i or Smad4-i cells were treated with TGF-β1 for 48 hr as indicated.

(H) Schematic representation of TSC1 mediated insulin-Akt -TGF-β crosstalk.

IB analysis of cell lysates (C, E, and G). ns, not statistically significant.

See also Figure S4.

TSC2 deficiency (Figure 4I) or by mTORC1 inhibition (Figure 4J). Thus, TSC1 mediates Akt-dependent Smad2/3 phospho-activation, independently of its function in the TSC1-TSC2 complex. Consistently, insulin stimulation also enhanced TGF-β1-induced Smad2/3 phosphorylation (Figure 4K), which was abolished in TSC1-deficient cells (Figure 4L). Furthermore, overexpression of phosphatase and tensin homolog (PTEN), a PI3K antagonist that counteracts Akt activation, inhibited pSmad2/3 (Figure S3E). Thus, TSC1 links insulin-PI3K-Akt signaling to TGF-β-Smad2/3 pathway activation.

To test if insulin-induced Smad2/3 phospho-activation involves mTORC1, insulin and TGF-β1 stimulation were combined with mTORC1 inhibition. Of note, enhanced Smad2/3 phosphorylation upon insulin treatment was even further increased in Raptor knockdown cells (Figure S3F). Inactivation of Raptor attenuates the mTORC1-dependent NFL, resulting in enhanced Akt activation. Thus, increased Smad2/3 phosphorylation in Raptor deficient cells may be due to Akt activation caused by NFL inhibition. In agreement, we observed strongly enhanced Akt-pT308 upon insulin stimulation in HeLa Raptor-i cells (Figure S3F).
Complementing these findings, TSC1-myc overexpression increased pSmad2/3 under rapamycin-induced Akt activation (Figure S3G). TSC1-myc expression without Akt activation did not affect Smad2/3 phosphorylation, consistent with a stimulatory function of Akt upstream of TSC1 in Smad2/3 regulation. This suggests that changes in mTORC1 activity, via the NFL, can indirectly control Smad2/3 phospho-activation when Akt is activated, e.g., by insulin.

TβR-I-mediated Smad2/3 phospho-activation takes place at the plasma membrane and in EEA1 positive early endosomes, containing Smad proteins and the internalized activated TGF-β receptor complex (Atfi et al., 2007, Chen, 2009, Di Guglielmo et al., 2003, Hayes et al., 2002, McLean and Di Guglielmo, 2010). Within the mTOR pathway, the TSC1- TSC2 complex localizes to lysosomes (Demetriades et al., 2014, Dibble et al., 2012, Menon et al., 2014) and peroxisomes (Zhang et al., 2013) to regulate the mTOR activator Rheb. Where does TSC1 exert its TSC2-independent function in TGF-β pathway regulation? In line with previous studies (Plank et al., 1998, Zhang et al., 2013), we observed that anti-TSC1 staining yields a punctate cytoplasmic pattern reminiscent of discrete vesicular structures (Figure 5A; specificity control for TSC1 antibody in Figure 5B). Co-staining of TSC1 with the early endosome marker EEA1 revealed an overlap (Figure 5A; negative control in Figure 5C), quantified by the nonlinear Spearman’s rank correlation coefficient \( r_s \) \( (r_s = 0.51 \pm 0.13 \text{ SD}) \). In keeping with the stimulatory insulin-Akt function upstream of TSC1 (Figures 4G, 4H, 4K, and 4L), insulin stimulation, but not TGF-β1, altered the pattern of TSC1-EEA1 co-localization structures (Figures 5D–5F), and significantly increased their co-localization \( (r_s \text{ starved} = 0.36 \pm 0.05 \text{ SD}; r_s \text{ insulin} = 0.49 \pm 0.01 \text{ SD}; r_s \text{TGF-β1} = 0.40 \pm 0.03 \text{ SD}; p \text{ starved versus insulin} < 0.01; p \text{ starved versus TGF-β1} > 0.05) \), without increasing total TSC1 protein levels (Figure 5G).

**TSC1 Is Required for TGF-β1 Induced Growth Arrest and EMT**

TGF-β-Smad signaling is a major regulator of growth arrest and EMT (Massagué, 2012). Hence, we tested whether TSC1 and Akt regulate Smad-dependent features of these processes. We used epithelial NMuMG cells as a well-established cell model to study the cellular TGF-β response (Deckers et al., 2006, Lamouille and Derynck, 2007, Miettinen et al., 1994, Piek et al., 1999, Valcourt et al., 2005).

TGF-β causes growth arrest in the G1 phase (Bhowmick et al., 2003, Lamouille and Derynck, 2007, Law et al., 2002, Miettinen et al., 1994, Shin et al., 2001, Valcourt et al., 2005), and interference with Smad signaling, e.g., by Smad4 knockdown, results in impaired growth arrest (Levy and Hill, 2005, Zhang et al.,...
TSC1 Activates TGF-β-Smad2/3 Signaling

1996). Consistent with earlier reports (Bhowmick et al., 2001, Levy and Hill, 2005, Zhang et al., 1996), TGF-β1 treatment resulted in an increased percentage of cells in the G1 phase in NMuMG Ctrl-i cells (Figures 6A and S4A). This induction of G1 arrest was significantly reduced in Smad4 knockdown cells (Figures 6A and S4A). TSC1 deficiency significantly impaired TGF-β1-induced growth arrest in G1 phase, whereas TSC2 knockdown did not alter TGF-β1-dependent growth arrest (Figures 6A and S4A). Similar to our previous findings in HeLa and HEK293T cells, NMuMG cells displayed reduced TGF-β1-dependent Smad2/3 phosphorylation in TSC1, but not in TSC2, knockdown cells (Figure 6B) and enhanced Smad2/3 phosphorylation upon myr-Akt expression (Figure 6C), suggesting comparable crosstalk mechanisms in NMuMG cells. Consistently, myr-Akt triggered G1 arrest in NMuMG Ctrl-i cells (Figure 6D) and this was suppressed by Smad4 deficiency (Figure 6D). Taken together, these results are consistent with a stimulatory function of hyperactive Akt and TSC1 in cytostatic Smad signaling in NMuMG cells.

Besides its cytostatic action, TGF-β is a crucial inducer of EMT. EMT is fundamental in organismal development and cancer metastasis, and contributes to the tumor-promoting function of TGF-β signaling in later stages of cancer development (Wendt et al., 2012). TGF-β-dependent EMT is mediated by Smad and non-Smad pathways, which control a complex transcriptional program, ultimately resulting in loss of epithelial markers and induction of mesenchymal proteins (Derynck et al., 2014). Are Smad-dependent EMT features regulated by TSC1 and Akt? In accordance with earlier observations (Deckers et al., 2006), up-regulation of the mesenchymal proteins N-Cadherin and Fibronectin in response to TGF-β1 was Smad4-dependent (Figure 6E). Importantly, TSC1 deficiency strongly impaired N-cadherin and Fibronectin induction by TGF-β1, whereas the expression of both target genes was not inhibited by TSC2 deficiency (Figure 6E). Furthermore, TSC1, but not TSC2, deficiency abolished Smad4-dependent morphological changes (Piek et al., 1999, Valcourt et al., 2005) in response to TGF-β1 treatment, including cellular transformation to a spindle-shaped morphology, loss of intercellular contacts, and formation of stress fibers (Figures 6F and S4B). Thus, TSC1 is not only required for TGF-β1-induced growth arrest but also for TGF-β1-induced EMT. We next tested whether Akt also affects TGF-β-dependent EMT in our cell system. Depending on the physiological context, Akt1 has been shown to promote (Bakin et al., 2000, Ju et al., 2007, Xue et al., 2012, Zhang et al., 2012, Zhou et al., 2006) or inhibit (Chin and Toker, 2010, Dillon et al., 2009, Hutchinson et al., 2004, Iliopoulos et al., 2009, Irie et al., 2005, Liu et al., 2006, Maroulakou et al., 2007, Yoeli-Lerner et al., 2005) EMT,
migration, and metastasis. We found that in NMuMG cells, myr-Akt1 counteracted TGF-β1-induced downregulation of the epithelial marker E-cadherin (Figure 6G) and did not affect morphological EMT changes (Figure S4C). Thus, despite its stimulating effect on Smad2/3 phosphorylation (Figure 6C), myr-Akt did not reinforce or accelerate TGF-β1-induced transcriptional or morphological EMT features (Figures 6G and S4C). The same stably myr-Akt expressing cell line showed enhanced Smad-dependent G₁ arrest (Figure 6D), confirming that myr-Akt expression was sufficient to trigger a physiological response. Different direct and indirect Akt1 targets have been implicated in either the stimulatory (Ju et al., 2007, Xue et al., 2012, Zhang et al., 2012), or the inhibitory (Chin and Toker, 2010, Irie et al., 2005, Liu et al., 2006, Yoeli-Lerner et al., 2005) function of Akt1 in EMT and metastasis. Thus, one may hypothesize that Akt activity concomitantly provides both stimulating and inhibiting inputs on EMT by regulating distinct sub-populations of its substrates. Thus, in NMuMG cells, the stimulatory input of myr-Akt1-dependent Smad phosphorylation (Figure 6C) may be overridden by inhibitory inputs, preventing the acquisition of a mesenchymal phenotype (Figure S4C).

**DISCUSSION**

We identify TSC1 as a component of the TGF-β-Smad2/3 pathway that positively regulates TβRI association with its substrates Smad2/3 (Figure 3E) and thus stimulates Smad2/3 phospho-activation (Figures 1A–1C, 6B, S1A, S1C, and S1D) (scheme depicted in Figure 6H). TSC1 exerts this function independently of TSC1-TSC2 complex activity (Figures 1A, 1B, 1D, 6B, and S1D). In keeping with this, TSC1 (but not TSC2) is required for both Smad-dependent growth arrest and EMT (Figures 6A, 6E, and 6F) and thus contributes to processes underlying both the tumor-suppressive and the pro-metastatic action of the TGF-β-Smad pathway. Insulin-Akt promotes Smad2/3 phosphorylation in a TSC1-dependent manner (Figures 4G, 4H, 4K, and 4L), and hyperactive Akt triggers cytostatic Smad signaling to induce growth arrest (Figure 6D). Thus, TSC1 links insulin-Akt signaling to the TGF-β-Smad2/3 pathway.

What may be the physiological function of this crosstalk? A hyperactive PI3K-Akt axis is a serious threat for an organism, as unrestrained Akt-dependent cell growth and proliferation may result in tumor development (Zoncu et al., 2011). It is well conceivable that in contexts of aberrant Akt activity, TSC1-dependent TGF-β-Smad activation represents a rescue mechanism to prevent cellular overgrowth via induction of cytostatic target genes and growth arrest (Figures 1H, 6B,6D, S1E,
TSC1 Activates TGF-b-Smad2/3 Signaling

S1F, and S4A). Such a cytostatic function of Akt seems to be at odds with Akt’s established role as a survival kinase (Hers et al., 2011). However, several recent studies have revealed that whereas Akt activity normally stimulates cell proliferation and survival, aberrantly activated Akt may in contrast induce apoptosis and cellular senescence, a state of permanent cell-cycle arrest (Los et al., 2009, Minamino et al., 2004, Miyauchi et al., 2004, Nogueira et al., 2008). We propose here that Akt-TSC1-TGF-β crosstalk represents one molecular mechanism that accounts for the cytostatic action of hyperactive Akt.

Of note, two previous studies (Conery et al., 2004, Remy et al., 2004) have reported that Akt directly interacts with the Smad3 protein to counteract Smad3 phospho-activation and Smad3-mediated apoptosis, in cell types (Hep3B, Ba/F3) other than those used in our study (NMuMG, HeLa, HEK293T). More recently, however, Zhang et al. (2012) reported a stimulatory function of Akt on Smad2/3 signaling in different breast cancer cell lines. Here, Akt phospho-activated the deubiquitylating enzyme USP4, resulting in reduced ubiquitylation-mediated degradation of TβR-I and thus enhanced Smad signaling (Zhang et al., 2012). Moreover, in a study of Xue et al. (2012), Akt has been shown to transcriptionally upregulate TGF-β2 in MDCK and 4T1 cells via phospho-activation of the transcription factor Twist1. This resulted in enhanced Smad2 signaling (Xue et al., 2012). Importantly, in vivo evidence exists for both inhibitory (Duenker et al., 2005) and stimulatory (Xue et al., 2012, Zhang et al., 2012) inputs of insulin/Akt on TGF-β-Smad signaling. Taken together, these and our findings suggest that multiple mechanisms of Akt-TGF-β crosstalk exist. It is conceivable that even opposing molecular crosstalk mechanisms may take place simultaneously in a cell. The net outcome of Akt-dependent TGF-β pathway regulation, i.e., inhibition or stimulation, would thus depend on the specific physiological condition.

Smad signaling exerts fundamental functions during embryonic development. Smad2−/− mice display gastrulation and mesoderm formation defects and die before E8.5 (Heyer et al., 1999, Nomura and Li, 1998, Waldrip et al., 1998, Weinstein et al., 1998). Interestingly, TSC1−/− mice progress normally through gastrulation and die only at later stages of embryonic development (embryonic day 10.5 [E10.5]–E11.5) (Kobayashi et al., 2001). What accounts for the phenotypic differences observed between Smad2−/− and TSC1−/− mice? Similar to TSC1 (Figures 5A–5F), multiple other modulators of Smad2/3 phospho-activation reside at the early endosomes, including SARA (Tsukazaki et al., 1998), cytoplasmic PML (cPML) (Lin et al., 2004), endofin (Chen et al., 2007), and HGF-regulated tyrosine kinase substrate (Hgs/Hrs) (Miura et al., 2000). All existing knockout mouse
models of the previously mentioned Smad modulators (Komada and Soriano, 1999, Wang et al., 1998) fail to recapitulate important features of Smad2−/− mice, such as lack of mesoderm, gastrulation defects, and early embryonic lethality. This raises the possibility that different endosomal regulators can compensate for each other in Smad activation, which may also explain why TSC1−/− mice do not recapitulate Smad2 knockout features.

TSC1 ablation results in phenotypes that closely resemble TSC2 mutants in different organisms (Orlova and Crino, 2010). This, together with the well-established TSC1-TSC2 interaction, led to the general view that TSC1 and TSC2 act exclusively in complex. Our results, and previous findings (Miloloza et al., 2002), challenge the common notion that TSC1 and TSC2 are strictly interdependent. Further evidence for separate functions of TSC1 and TSC2 comes from microarray analyses and proteomic approaches, which reveal that the TSC genes trigger substantially different cellular responses (Hengstschläger et al., 2005, Rosner et al., 2005). Interestingly, in some cancers (renal, bladder) TSC1 mutations seem to be more prevalent, as compared to TSC2 (Hornigold et al., 1999, Kucejova et al., 2011, Pymar et al., 2008). How can a TSC2-independent function of TSC1 be reconciled with the highly similar phenotypes of TSC1 and TSC2 mutants? Inactivation of TSC1 or TSC2 strongly induces mitogenic mTORC1 signaling; one may hypothesize that constitutive aberrant mTORC1 activation due to severe TSC1 or TSC2 dysfunction overrides more subtle regulatory mechanisms and might thus eventually dominate the phenotypes caused by TSC1 or TSC2 mutations. However, when the TSCs are functional, the TSC1-TGF-β crosstalk may play an important physiological role particularly under conditions of active Akt signaling.

Our findings have critical implications for cancer therapy, because drugs inhibiting Akt could have the unfavorable side effect of abrogating cytostatic TGF-β signaling. On the other hand, mTORC1 inhibitors may exert their growth-inhibitory function in part by activating Akt (via the NFL) and TSC1-dependent cytostatic TGF-β signaling. This suggests that the TGF-β network should be cautiously monitored upon treatment with PI3K, Akt, and mTOR inhibitors, and that the contribution of the TGF-β pathway to the treatment outcome of these drugs warrants investigation in clinical studies.
EXPERIMENTAL PROCEDURES

Cell Culture, Drug Treatments, Cloning, and Transfection
HeLa, HEK293T, and NMuMG cells were cultured according to the recommendations of the American Type Culture Collection (ATCC). Cloning, transfection, treatments, and plasmids (including those received from Addgene) are described in detail in the Supplemental Experimental Procedures. Addgene plasmids used in the present study were originally described in the following publications: Cai et al., 2006, Ramaswamy et al., 1999, Tee et al., 2002, Wrana et al., 1992, Zhang et al., 1998). C-terminally myc-tagged TSC1 full-length and truncation constructs were a kind gift of Mark Nellist, Erasmus MC, Rotterdam, the Netherlands, and published in Hoogeveen-Westerveld et al. (2010).

RNA Interference and Transgenic Cell Lines
Cell lines with inducible knockdown of TSC1, TSC2, Rheb, and Raptor were generated using the previously described doxycycline/tetracycline-sensitive tTR-KRAB system (Wiznerowicz and Trono, 2003). Details on transduction, knockdown induction, and target sequences of shRNA are given in the Supplemental Experimental Procedures.

NMuMG control and Smad4 knockdown cells were a kind gift from Prof. Gerhard Christofori (University of Basel, Switzerland) and previously described in Deckers et al. (2006).

Cell Lysate Preparation, Immunoprecipitation, Immunoblot, and Quantitation
Cell lysis, immunoprecipitation (IP), and immunoblot (IB) analysis were performed as described (Dalle Pezze et al., 2012). Details on lysis buffers, IB analysis, and antibodies used in IB and IP are given in the Supplemental Experimental Procedures.

Immunofluorescence, Imaging, and Quantitation
Cells were cultivated on glass coverslips in six-well tissue culture plates. General staining procedures were performed as described (Thedieck et al., 2013). See Supplemental Experimental Procedures for details on staining protocols, wide field and confocal imaging, and image analysis. Antibodies used in IF are listed in the Supplemental Experimental Procedures (list of antibodies).
Chapter 6

Proximity Ligation Assay
The PLA technique was performed as previously described (Söderberg et al., 2008). All reagents used for PLA analysis were purchased from Olink Bioscience, Sweden (current distributor: Sigma-Aldrich). Experimental details and antibodies used in PLA are described in the Supplemental Experimental Procedures.

Propidium Iodide Staining and Flow Cytometry
Cell-cycle analysis was performed using propidium iodide staining, followed by fluorescence-activated cell sorting analysis. For details, see the Supplemental Experimental Procedures.

Statistical Analysis
The following experiments involving control and TSC1/2 knockdown cells under different treatments were statistically analyzed with one-way ANOVA followed by Tukey’s Multiple comparison test (suitable for statistical comparison of multiple experimental groups): pSmad/total Smad ratio (Figures 1B, 2B, 2E, and 6B), nuclear localization of pSmad2/3 (Figure 1G), expression of p57Kip2 (Figure S1F), TSC1-Smad2/3 association (Figure 3B), and G1 arrest (Figures 6A and 6D). Two-tailed Student’s t test assuming unequal variances was used for statistical analysis of TSC1-TβR-I and TβR-I-Smad2/3 interaction in myr-Akt/empty vector transfected HeLa cells (Figures 4B and 4E), and TSC1-EEA1 co-localization quantified by the Spearman’s correlation coefficient (see manuscript text).

p values above 0.05 were considered non-significant.

Author Contributions
A.T. planned and conducted experiments, analyzed the results, and wrote the manuscript; M.T.P. planned and conducted experiments, analyzed the results, and contributed to manuscript writing; B.H. planned and conducted experiments and analyzed the results. C.B. performed confocal microscopy; I.K., A.G.S., S.R., and L.M. contributed to experimentation; R.N. supported confocal microscopy and imaging analysis; S.N.G. designed the strategy for TSC1 mutagenesis; M.R. and K.K. enabled and conducted PLA and FACS analyses; R.B. and G.W. supported the manuscript preparation; R.B. and E.N.-H. contributed to project guidance and manuscript preparation; and K.T. planned and guided the project, planned and analyzed the experiments, and wrote the manuscript.
Acknowledgments
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Figure S1, related to Figure 1.

**TSC1 regulates TGF-β - Smad2/3 signaling, independently of TSC2.**

(A) Reduced Smad phosphorylation in HeLa TSC1-i #1 and TSC1-i #2 upon long term TGF-β1 stimulation. HeLa Ctrl-i, TSC1-i #1 and TSC1-i #2 shRNA cells were stimulated with TGF-β1 as indicated.

(B) Expression level of TSC1-myc wild type (TSC1-mycwt), but not of a TSC1-myc construct mutagenized within the TSC1-i #1 binding region (TSC1-mycnon-targeted), is reduced upon induction of TSC1-i #1 shRNA expression. HeLa TSC1-i #1 and Ctrl-i cells were transfected for 48 h with TSC1-mycwt or TSC1-mycnon-targeted as indicated.

(C) Deficient Smad phosphorylation in TSC1-i #1 knockdown cells is restored upon TSC1-mycnon-targeted overexpression. HeLa TSC1-i #1 and Ctrl-i cells were transfected and stimulated with TGF-β1 (24 h) as indicated. All cells were incubated with rapamycin to exclude potential indirect mTORC1-mediated effects of TSC1-mycnon-targeted on Smad phosphorylation.

(D) TSC1 is required for TGF-β1 induced Smad2/3 phosphorylation in HEK293T cells. Inducible HEK293T Ctrl-i, TSC1-i and TSC2-i shRNA cells were treated with doxycycline to induce shRNA expression, or left untreated. Cells were starved and stimulated with TGF-β1 for 45 min as indicated. (E) TSC1, but not TSC2, is required for TGF-β1 induced expression of p57Kip2. HeLa Ctrl-i, TSC1-i and TSC2-i shRNA cells were stimulated with TGF-β1 for 48 h as indicated.

(F) Quantitation of p57Kip2 levels of three independent experiments as performed in (E); ratio of p57Kip2 levels of TGF-β1 treated cells / p57Kip2 levels of untreated cells. Results are shown as box plots representing median, 25th and 75th percentiles as boxes, and the range of data as bars; one-way ANOVA; *, p < 0.05.

IB analysis of cell lysates (A-E). ns = not statistically significant.
### TSC1 Activates TGF-β-Smad2/3 Signaling

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Figure S2, related to Figure 3.
TSC1 interacts with TGF-β receptor proteins and Smad2/3.

(A) Protein levels of core TGF-β pathway components. IB analysis of the same cell lysate samples which are shown in Figure 1A (inducible HeLa Ctrl-i, TSC1-i and TSC2-i shRNA cells +/- doxycycline, starved and stimulated with TGF-β1 for 45 min as indicated).

(B) TSC1 – Smad2/3 interaction. HeLa cells were transfected with TSC1-myc and Flag-Smad2 or Flag-Smad3, respectively. IP was performed using either Flag-specific or control mouse IgG antibodies ('mock').

(C) TSC1 specifically interacts with Flag-Smad3 and does not unspecifically bind to anti-Flag immunobeads. HeLa cells were transfected with TSC1-myc, and either Flag empty vector or Flag-Smad3, respectively. IP was performed using Flag-specific antibody.

(D) N-terminal domain of TSC1 is required for Smad3 interaction. HeLa cells were transfected with the indicated TSC1 constructs or with empty vector and IP was performed using Flag-specific antibody.

(E) TSC1 – TβR-I association. HeLa cells were stimulated with TGF-β1 for 10 min. Confocal images of TSC1 – TβR-I PLA and negative controls with single primary antibodies.

(F) TSC1 – TβR-II interaction. HeLa cells were transfected with TSC1-myc. IP was performed using either TβR-II-specific or control rabbit IgG antibodies ('mock').

(G) TSC1 – TβR-I interaction does not require TSC2. HeLa Ctrl-i and TSC2-i shRNA cells were transfected with TSC1-myc and IP was performed using either TβR-I-specific or control rabbit IgG antibodies ('mock').

(H) Endogenous TβR-I – Smad2/3 association at 40 min of TGF-β1 stimulation is TSC1-dependent. HeLa Ctrl-i and TSC1-i shRNA cells were starved and stimulated for 40 min with TGF-β1. Confocal images of PLAs.

Scale bar: 10 μm (E, H)

IB analysis of cell lysates (A-D, F, G) and IPs (B-D, F, G).
Figure S3, related to Figure 4.

TSC1 links insulin - Akt activity to the TGF-β pathway.

(A) Constitutively active myr-Akt reduces TSC2, but not TSC1 protein levels. HeLa cells were transfected with myr-HA-Akt or empty vector (indicated amounts per 6 cm dish).

(B) Binding of endogenous TSC1 to TSC2 is decreased by myr-Akt. HeLa cells were transfected with myr-HA-Akt or empty vector as indicated. IP was performed using either TSC1-specific or control mouse IgG antibodies (‘mock’).

(C) Constitutive Akt activity (myr-Akt) does not enhance TGF-β1 induced Smad phosphorylation in presence of the TβR-I inhibitor SB431542. HeLa cells were transfected with myr-HA-Akt or empty vector, starved, and treated with SB431542 (10 μM, 1 h) prior to TGF-β1 stimulation (10 min) as indicated.

(D) Enhanced Smad phosphorylation upon constitutive Akt activity is TSC1-dependent. Inducible HeLa Ctrl-i and TSC1-i shRNA cells were treated with doxycycline to induce shRNA expression, or left untreated. Cells were transfected with myr-HA-Akt or empty vector as indicated and cultivated in full medium prior to lysis.

(E) PTEN overexpression (inhibiting Akt) impairs TGF-β1 induced Smad2/3 phosphorylation. HA-PTEN or empty vector transfected HeLa cells were starved, treated with insulin (100 nM, 40 min), and stimulated with TGF-β1 as indicated.

(F) Raptor knockdown further enhances Smad2/3 phosphorylation induced by insulin stimulation. HeLa Ctrl-i and Raptor-i shRNA cells were starved and treated with insulin (100 nM, 4 h) as indicated prior to TGF-β1 stimulation (15 min).

(G) TSC1 overexpression and rapamycin (inducing Akt) synergistically regulate Smad2/3 phosphorylation. HeLa cells (Ctrl-i with doxycycline) were transfected with TSC1-myc or empty vector, pretreated with rapamycin (100 nM, 1 h) and stimulated with TGF-β1 for 15 min as indicated.

IB analysis of cell lysates (A-G) and IP (B).
Figure S4, related to Figure 6.
Akt and TSC1 in TGF-β1 induced growth arrest and EMT.

(A) TSC1 deficiency impairs TGF-β1 induced G1 growth arrest in NMuMG cells. NMuMG Ctrl-i, TSC1-i, TSC2-i and Smad4-i shRNA cells were treated with TGF-β1 (24 h) and subjected to propidium iodide staining followed by flow cytometry. Shown are the percentages of cells in G1. Quantitation of four independent experiments with each condition being performed in duplicates or triplicates in each of the four independent experiments. Mean values +/- SD.

(B) TSC1 deficiency impairs EMT in response to TGF-β1 treatment (72 h). Bright field images of NMuMG Ctrl-i, TSC1-i, TSC2-i and Smad4-i shRNA cells.

(C) Myr-HA-Akt does not enhance morphological EMT changes in response to TGF-β1 treatment. Bright field images of NMuMG cells transduced with myr-HA-Akt or empty vector and treated with TGF-β1 (48 h, two concentrations) as indicated. Scale bar (B,C): 60 µm.
SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Treatments, cloning and transfection

Starvation was performed overnight in serum-free DMEM (4.5 g glucose/L; supplemented with 1.5 % (v/v) glutamine, Life Technologies, USA, #25030081). In insulin stimulation experiments, cells were starved in HBSS for growth factors and amino acids to obtain efficient silencing of the insulin - Akt - mTORC1 signaling axis. TGF-β1 stimulation: 5 ng/mL TGF-β1 (CHO cell derived, Peprotech, Germany) in serum-free DMEM, times are indicated in the figure legends. Insulin stimulation: 100 nM insulin (Sigma-Aldrich, USA, #I1882) in serum-free DMEM, 2-4 h. Pre-treatment with rapamycin (Calbiochem, Germany) / PP242 (Sigma Aldrich, USA): 200 nM; 1 h prior to lysis or TGF-β1 stimulation (in Figure S3G, 100 nM rapamycin was used). SB431542 (Sigma-Aldrich, USA): 10 μM; 1 h prior to TGF-β1 stimulation. BMP4 stimulation: 10 ng/mL (Recombinant Human BMP-4, Life Technologies, USA). Transfection was performed using JetPEI (Polyplus, France) according to the manufacturer’s instructions and transfected cells were harvested 24-48 h post-transfection. For plasmids, see list of plasmids.

To create a functional TSC1-myc construct that is no longer targeted by TSC1-i #1 (TSC1-mycnon-targeted), three silent mutations were introduced into the TSC1-i #1 binding site of C-terminally myc tagged pcDNA3.1.TSC1 (wild type sequence: GACACACAGAATAGCTATG; mutagenized sequence: GACACtCAGAAcAGCTAeG; mutagenized nucleotides are underlined and in bold small letters). This was done in three sequential mutagenesis PCR rounds using Agilent Technologies QuickChange II Site directed mutagenesis kit XL (Stratagene, Agilent, Santa Clara, CA, USA, #200521).

RNA interference and transgenic cell lines.

For inducible knockdown of TSC1, TSC2, Rheb, and Raptor, HeLa, HEK293T, and NMuMG cells were first transduced with lentivirus encoding the doxycycline/tetracycline-sensitive tTR-KRAB repressor and a DsRed reporter. In a second step, cells were transduced with lentivirus encoding the specific shRNA or non-targeting control shRNA and a GFP reporter (pLVTH vector), which are both under the control of tTR-KRAB. To induce the expression of shRNA, cells were treated with 2 μg/mL doxycycline for 3-5 days. For target sequences of shRNA see list of shRNA sequences. For better comparability, stable NMuMG Smad4 knockdown cells were
virus transduced with non-targeting scramble shRNA in parallel to the generation of NMuMG TSC1-i, TSC2-i, and Ctrl-i, and all cell lines were treated with doxycycline prior to experiments. For experiments involving NMuMG cells stably expressing myr-Akt, NMuMG cells were transduced with lentiviruses encoding pLNCX.myr-HA-Akt or empty vector (Figure 6D, G, and S4C).

**Cell lysate preparation, immunoprecipitation (IP), immunoblot (IB), and quantitation.**

Antibodies are listed below. Whole cell lysate preparation was performed in RIPA buffer. Proteins for IP experiments were extracted in IP buffer (50 mM Tris-Cl [pH 7.4], 150 mM NaCl, 3 mM MgCl2, 1 mM CaCl2, 1 % (w/v) CHAPS; special buffer for IP of endogenous TSC1: 40 mM HEPES [pH 7.4], 120 mM NaCl, 0.3 % (w/v) CHAPS). All buffers were supplemented with protease and phosphatase inhibitors. IPs were performed as previously described (Dalle Pezze et al., 2012) using DynaBeads® Protein G (Invitrogen, USA). For detection of immunoprecipitated proteins, Clean-Blot IP Detection Reagent (HRP) (Thermo-scientific, USA) was used. Immunoblot signals were detected using a LAS-4000 mini camera system (Fujifilm Life Science Systems, Japan). Signal intensities were assessed using Multi Gauge V3.0 software (Fujifilm Life Science Systems, Japan). For pSmad / total Smad quantitation, signal intensities of Ctrl-i cells were set to ‘1’ and signal intensities of knockdown cells were normalized accordingly. For statistical analysis, three independent experiments were quantified and analyzed.

**Immunofluorescence (IF), imaging, and quantitation.**

**IF staining of Smad2/3 in HeLa cells:**

Cells were fixed for 15 min in 4 % paraformaldehyde in PBS and incubated with anti-Smad2/3 antibody (Cell Signaling #5678; overnight, 4 °C), followed by a Cy5® labeled secondary antibody (see antibodies listed below). Cells were mounted with ProLong Gold Antifade (Dapi included). Confocal imaging was performed using a LSM 510 DUO laser scanning microscope equipped with a 100x/1.4 Plan-Apochromat oil DIC objective (Carl Zeiss Microscopy, Germany). Images were taken using a frame size of 732 × 732 pixels. Laser power, amplifier offset and detector gain were adjusted first and never changed for an experimental series. For semi-quantitative analysis of nuclear versus cytoplasmic fluorescent signal of Smad2/3, at least five independent fields of view from three independent experiments for each condition (Ctrl-i no TGF-β1, Ctrl-i + SB431542, Ctrl-i + TGF-β1, TSC1-i + TGF-β1, TSC2-i + TGF-β1) were analyzed. Each cell was assigned to one of two groups: “Smad2/3 predominantly in the nucleus” or “cytoplasmic Smad2/3 distribution”.

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**TSC1 Activates TGF-b-Smad2/3 Signaling**
IF staining of TSC1 and EEA1 in HeLa cells:
HeLa cells were fixed in 4 % paraformaldehyde in PBS and incubated with anti-TSC1 (Invitrogen) and anti-EEA1 antibodies (overnight, 4 °C), followed by Hoechst 33342 and Alexa Fluor® 568 and Alexa Fluor® 488 labeled secondary antibodies (see antibodies listed below).

Confocal imaging was performed using a LSM 510 DUO laser scanning microscope equipped with a 100x/1.4 Plan-Apochromat oil DIC objective (Carl Zeiss Microscopy, Germany). Z-stack images were taken using a frame size of 1024 × 1024 pixels. Sequential acquisition of the different channels was performed. Laser power, amplifier offset and detector gain were adjusted first and never changed for an experimental series. Three experiments were performed, and four fields of view were acquired for each experiment and condition (full medium, starved, insulin, TGF-β1) and subjected to further analysis. Raw images were deconvoluted using the Huygens Core software (Scientific Volume Imaging, Netherlands). To quantify the extent of co-localization of anti-TSC1 and anti-EEA1 staining, deconvoluted images were analyzed for the Spearman’s rank correlation coefficient using the Huygens Professional software (Scientific Volume Imaging, The Netherlands) and applying the “optimised background” subtraction mode. For better visibility, images displayed in the manuscript were processed with Imaris 7.7.2 software (BITPLANE AG, Switzerland), using the same settings in each channel for all experimental conditions. Image processing does not affect quantitation of TSC1 – EEA1 co-localization. Images of one representative replicate are shown.

IF staining of TSC1 and myc in TSC1-myc transfected HeLa cells:
Transfected HeLa cells were fixed in methanol and co-stained with anti-TSC1 (Invitrogen) and anti-myc antibodies (overnight, 4 °C), followed by Hoechst 33342 and Alexa Fluor® 488 and Alexa Fluor® 568 labeled secondary antibodies (see antibodies listed below). Wide field fluorescence imaging was performed using an Axio-Imager Z.1 microscope (Carl Zeiss Microscopy, Germany) equipped with an ApoTome and an AxioCam MRm (Carl Zeiss Microscopy, Germany).

IF staining of actin in NMuMG cells:
NMuMG cells were fixed in methanol and incubated with anti-actin (1 h, room temperature), followed by an Alexa Fluor® 488 labeled secondary antibody (see antibodies listed below). Wide field fluorescence imaging was performed using an Axio-Imager Z.1 microscope (Carl Zeiss Microscopy, Germany) equipped with an ApoTome and an AxioCam MRm (Carl Zeiss Microscopy, Germany).
Proximity Ligation Assay (PLA)

The PLA technique was performed as previously described (Soderberg et al., 2008). All reagents used for PLA analysis were purchased from Olink Bioscience, Sweden (current distributor: Sigma Aldrich, USA). Briefly, cells were seeded on Teflon coated PLA slides (Menzel-Gläser, Thermo Scientific) and cultured for two days at 37 °C in 7.5 % CO2. Cells were fixed with 100 % methanol at -20 °C for 5 min. After fixation cells were treated with 0.5 % (w/v) Saponin in PBS for 15 min at 4 °C and 15 min at RT, and blocked in 5 % (w/v) BSA in PBS for 60 min at 37 °C. Cells were incubated with primary antibodies (see antibodies listed below) overnight at 4 °C. Next day, cells were incubated with the according PLA probes (secondary antibodies conjugated to unique DNA probes for anti-mouse, anti-rabbit or anti-goat) for 60 min at 37 °C. For ligation and circularization of the DNA-oligos, cells were incubated with ligase-solution for 30 min at 37 °C. For rolling circle amplification cells were incubated with amplification-solution, containing a complementary Alexa 555-labeled DNA linker as detectable fluorophore for 120 min at 37 °C. Cells were mounted on a cover slip using Duolink In Situ Mounting Medium with DAPI and analyzed by confocal microscopy (LSM 510 or LSM 780 META laser scanning microscope equipped with a 63x/1.4 Plan-Apochromat oil DIC objective; Carl Zeiss Microscopy, Germany). Pictures were taken with optimal frame size of 1024 x 1024 (1764 x 1764) pixels, scan speed 7 for superior images and with 12 bit (8 bit) pixel depth. Amplifier offset and detector gain were adjusted first and never changed for an experimental series. The signal-per-cell ratio was analyzed with ImageJ software (National Institute of Mental Health, USA), using the ‘Analyze Particles’ function. For semi-quantitative PLA analysis, ≥ 60 cells from ≥ three fields of view for each condition of one experiment were analyzed. Results are representative for three biological replicates.

Propidium iodide (PI) staining and flow cytometry

Cells from one 6 cm plate were trypsinized, washed 1x with PBS, and resuspended in 500 μL PBS containing 0.1 % glucose. For fixation, 5 mL ice-cold 70 % ethanol in PBS was added while gently vortexing the cells. Cells were kept at 4 °C for 1 h to 3 days. Cells were washed 1x with PBS and resuspended in 300 μL freshly prepared propidium iodide buffer (50 μg/mL propidium iodide in PBS), supplemented with RNase (400 μg/mL). RNA was digested 30 - 45 min at 37 °C. PI-staining was analyzed by FACS measurement with FACS-fortessa (BD Biosciences, USA). For each experiment 10000 cells were excited using a 488 nm laser. Red fluorescence emission was measured at 610 nm.
## List of Plasmids

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<th>Details</th>
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<td>N-terminally Flag tagged pRK5F-Smad2</td>
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<tr>
<td>N-terminally Flag tagged pRK5F-Smad3</td>
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<tr>
<td>C-terminally HA tagged pCMV5B.TβR-II</td>
<td>Addgene, USA, #11766</td>
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<tr>
<td>N-terminally V5 tagged pcDNA6.TβR-I</td>
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<td>C-terminally myc tagged pcDNA3.1.TSC1</td>
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<td>C-terminally myc tagged pcDNA3.1.TSC1non-targeted</td>
<td>This construct is not knocked down by shRNA TSC1-i #1. For cloning details, please see Supplemental Experimental Procedures (Treatments, cloning and transfection).</td>
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<td>N-terminally Flag tagged pRK7-FLAG-TSC1</td>
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### List of shRNA sequences

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## List of Antibodies

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