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ORIGINAL ARTICLE

TGF-β-mediated activation of RhoA signalling is required for efficient ^{V12}HaRas and ^{V600E}BRAF transformation

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Transforming growth factor β-1 (TGF-β) acts as both a tumour suppressor and a tumour promoter in a context-dependent manner. The tumour-promoting activities of TGF-β are likely to result from a combination of Smad and non-Smad signalling pathways but remain poorly understood. Here we show that TGF-β-mediated activation of RhoA is dependent on the kinase activity of ALK5 and that continuous ALK5 activity maintains basal RhoA–ROCK signalling, cell morphology and actin dynamics in serum-starved rodent fibroblasts independently of Smad2, Smad3 and Smad4. In immortalized human diploid fibroblasts, we show that oncogenic rewiring by transduction of ^{V12}HaRas instigates regulation of RhoA–ROCK signalling through an autocrine TGF-β1–ALK5 pathway. Furthermore, we show that ALK5-mediated activation of RhoA is required for efficient ^{V12}HaRas, V-Raf and ^{V600E}BRAF transformation and ^{V12}HaRas-mediated anchorage-independent growth. These findings identify a new pro-oncogenic activity of TGF-β and indicate that tumours harbouring ^{V12}HaRas and ^{V600E}BRAF mutations may be susceptible to TGF-β signalling inhibitors.
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Introduction

Transforming growth factor β-1 (TGF-β) is the prototypical member of a family of pleiotropic cytokines that regulate many biological processes during development, wound healing and tissue homoeostasis. TGF-β acts as a tumour suppressor by negatively regulating cell growth, but can also act as a tumour promoter in a multifactorial manner (Pardali and Moustakas, 2007). TGF-β signals through a heterotetramer of two constitutively active type II receptors (TβRII) and two activin-like kinase 5 (ALK5) or type I receptors (Shi and Massague, 2003). TGF-β binds to the high-affinity TβRII, which recruits and activates ALK5, allowing it to directly phosphorylate the intracellular signal transduction molecules Smad2 and Smad3. These then complex with the co-Smad Smad4, accumulate in the nucleus and regulate target gene expression (Massague *et al*., 2005). TGF-β can also activate several non-Smad pathways in a tissue-specific manner. These include the kinases Jun N-terminal kinase, p38MAPK (p38 mitogen-activated protein kinase), ERK (extracellular signal-regulated kinase-1) and PI3K (phosphoinositide-3 kinase), and the small GTPases Ras, RhoA, Rac and Cdc42 (Moustakas and Heldin, 2005). The molecular mechanisms of the activation of non-Smad pathways remain largely unknown and it has been proposed earlier that these pathways may play a prominent role in the tumour-promoting effects of TGF-β, although direct evidence supporting this hypothesis remains to be found (Wakefield and Roberts, 2002).

TGF-β can activate RhoA signalling in several cell types (Bhowmick *et al*., 2001, 2003; Shen *et al*., 2001; Edlund *et al*., 2002; Vardouli *et al*., 2005; Chen *et al*., 2006). Rho GTPases are molecular switches that are inactive when GDP bound and active when GTP bound, where they propagate their signals through interaction with numerous downstream signalling effectors (Jaffe and Hall, 2005). The regulation of GTPase activity of Rho family proteins is controlled by the coordinated actions of Rho guanine nucleotide exchange factors (RhoA-GEFs), which catalyse the exchange of GDP for GTP, and Rho GTPase-activating proteins (RhoGAPs), which stimulate the intrinsic GTPase activity of the Rho proteins (Jaffe and Hall, 2005). The Rho GTPases are involved in numerous signal transduction pathways and act as regulators of the actin cytoskeleton, cell motility and transcription and are associated with progression to malignancy in several types of cancer (Sahai and Marshall, 2002). Specifically, RhoA activity is required

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for oncogenic transformation of mouse fibroblasts by ^{V12}HaRas (Qiu *et al*., 1995) and is selected for during transformation to counteract the cytostatic effects of chronic Ras–MAPK signalling by an as-yet elucidated mechanism (Olson *et al*., 1998; Sahai *et al*., 2001).

In this study we have investigated the mechanism of activation of RhoA by TGF-β. Utilizing the recently characterized ALK5 kinase inhibitor SB-431542 (Inman *et al*., 2002) we show that TGF-β-mediated RhoA activation is kinase dependent. We also show that ALK5 regulates basal RhoA activity in serum-starved rodent fibroblasts in a Smad-independent manner. In human diploid fibroblast cell lines, we find ALK5 activity is ligand-dependent and that this autocrine loop is coincident with transduction by oncogenic ^{V12}HaRas. Furthermore, we show that efficient ^{V12}HaRas- and ^{V600E}BRAF-mediated transformation relies on ALK5-driven RhoA activity and provides a mechanistic explanation for the high RhoA activity selection process that operates during ^{V12}HaRas transformation (Olson *et al*., 1998; Sahai *et al*., 2001).

Results

Rapid activation of RhoA by TGF-β is blocked by the ALK5 kinase inhibitor SB-431542

Activation of non-Smad signalling pathways by TGF-β contributes to its biological effects in a context-dependent manner (Derynck and Zhang, 2003; Moustakas and Heldin, 2005); however, the molecular mechanisms of activation of non-Smad pathways remain largely unknown. TGF-β may activate RhoA signalling in a biphasic manner involving a rapid activation within minutes of ligand addition followed by a second, potentially Smad-dependent wave of activation after hours of TGF-β stimulation (Bhowmick *et al*., 2001, 2003; Shen *et al*., 2001; Edlund *et al*., 2002; Vardouli *et al*., 2005; Chen *et al*., 2006). Rapid TGF-β-driven activation of RhoA has been described in Swiss-3T3 and mouse embryonic fibroblast (MEFs) (Shen *et al*., 2001; Vardouli *et al*., 2005), and we first investigated whether this also takes place in NIH3T3 cells. We observed a rapid and transient TGF-β-mediated activation of RhoA in serum-starved NIH3T3 cells as measured in Rhotekin RhoA-binding domain (RBD) pull-down assays (Figures 1a and b lanes 3–5). TGF-β activated RhoA within 5 min of stimulation and this preceded robust Smad activation, which was observable after 10 min of treatment by which time RhoA activity had already declined (Figure 1a).

We have earlier characterized SB-431542, a small-molecule competitive ATP kinase inhibitor of ALK5, ALK4 and ALK7 (Inman *et al*., 2002), and we used this inhibitor to determine the ALK5 kinase activity dependence of TGF-β-mediated activation of RhoA. Pretreatment with 10 μM SB-431542 for 15 min prevented the TGF-β-driven rapid activation of RhoA, indicating that this process is dependent on ALK5 kinase activity (Figure 1b, lanes 6–8). We also observed that 15 min of SB-431542 treatment significantly lowered the basal level of RhoA-GTP, suggesting that ALK5 kinase activity directly regulates basal RhoA activity in these cells (Figure 1b, lane 2). This effect was also demonstrable in another rodent fibroblast cell line, as we found that 15 min of SB-431542 treatment resulted in a concentration-dependent decrease in basal RhoA-GTP levels in serum-starved REF52 cells (Figure 1b).

ALK5 regulates basal RhoA–ROCK signalling, cell morphology and the actin cytoskeleton

RhoA can activate several effector proteins including the Rho-dependent kinases ROCKI and ROCKII (ROCK) (Jaffe and Hall, 2005). ROCK regulates actomyosin contractility by controlling Ser19 phosphorylation of myosin light chain 2 (MLC2), by inactivating MLC phosphatase and possibly by directly phosphorylating MLC2 itself (Jaffe and Hall, 2005). The levels of PO_4-MLC consequently act as a measure of RhoA–ROCK signalling. We observed a rapid concentration-dependent decrease of PO_4-MLC2 in response to SB-431542 in REF52 cells (Figure 1d). Inhibition of PO_4-MLC2 levels was readily observable with 5 μM SB-431542 treatment, and this further declined with 10 and 20 μM SB-431542 treatments and approached the level of inhibition reached after treatment with the ROCK inhibitor Y27632 (Uehata *et al*., 1997) (Figure 1d).

RhoA signalling regulates actin stress fibre formation and focal adhesion dynamics. Serum-starved REF52 cells exhibited prominent actin stress fibres and focal adhesion contacts as visualized by phalloidin staining and immunofluorescence with anti-vinculin antibodies (Figure 1e). In agreement with our RhoA-GTP and PO_4-MLC2 assays, we observed a rapid change in cell morphology after 15 min treatment with 10 μM SB-431542. This treatment was sufficient to dramatically reduce the number of focal adhesions and actin stress fibres (Figure 1e). These phenotypic changes were also observed after Y27632 treatment, indicating that basal ALK5 kinase activity regulates the RhoA–ROCK pathway, cell morphology and actin dynamics.

We next tested whether the decrease in RhoA–ROCK signalling and actin cytoskeleton disruption evident upon SB-431542 treatment was dependent on ALK5 inhibition using ALK5 null MEFs (Larsson *et al*., 2001). SB-431542 (10 μM) treatment of wild-type MEFs resulted in a decrease in PO_4-MLC2 levels and a loss of actin stress fibres similar to that observed after ROCK inhibition (Supplementary Figure 1a and b). In contrast, we observed no effects of SB-431542 treatment on ALK5 null MEFs even when they were treated with a higher dose of SB-431542 (50 μM) (Supplementary Figures 1a and c). These cells have a normal functional ROCK-MLC2 pathway as Y27632 treatment abrogated PO_4-MLC2 levels and actin structure. SB-431542 still maintains specificity for ALK5 at 50 μM concentrations, as it efficiently inhibited basal and TGF-β-induced phosphorylation of Smad2 and Smad1, but had no effect on BMP4-induced Smad1 activation (Supplementary Figure 2), which is mediated by the highly related kinases ALK3 and ALK6 (Chen *et al*.,

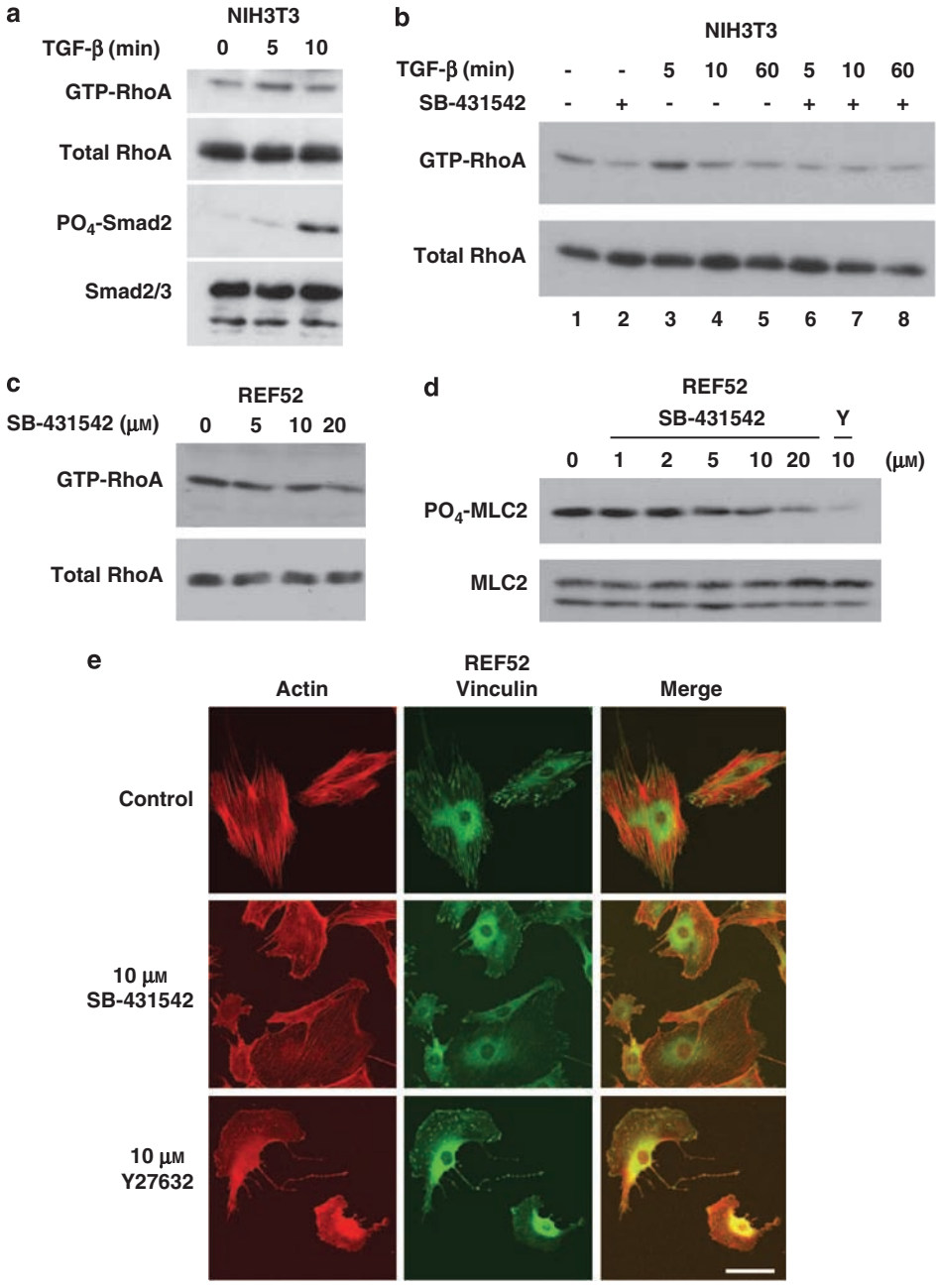

Figure 1 ALK5-dependent TGF-β signalling regulates basal RhoA–ROCK activity. (**a**) Serum-starved NIH3T3 cells were treated with 1 ng/ml TGF-β for the indicated time points. The level of active GTP-RhoA was determined by GST-rhotekin RBD pull-down analysis. Pull down and total cell lysates were analysed by western blotting with anti-RhoA antibodies. The levels of phosphorylated Smad2 (PO_4-Smad2) and total Smad2 were determined by western blotting. (**b**) Serum-starved NIH3T3 cells were pretreated for 15 min with 10 μM SB-431542 (+) or 0.1% DMSO carrier (−) and then with 1 ng/ml TGF-β for the indicated time points. The levels of total and GTP-RhoA were determined as in panel a. (**c**) Serum-starved REF52 cells were incubated with SB-431542 at the concentrations indicated for 15 min. The level of active GTP-RhoA was determined as described in panel a. (**d**) Serum-starved REF52 cells were treated with indicated concentrations of SB-431542 or Y27632 (Y) for 15 min, and the levels of total and phosphorylated MLC2 were determined by western blotting using anti-MLC2 and anti-phospho MLC2 (PO_4-MLC) antibodies. (**e**) Cells were treated as in panel d, fixed and analysed by immunofluorescence microscopy using anti-vinculin antibodies and tetramethylrhodamine α-isothiocyanate (TRITC)-phalloidin (actin). Scale bar indicates 20 μm.

2004). Furthermore, we observed that SB-431542 treatment disrupted actin stress fibres and focal adhesions in NIH3T3 cells, and activation of RhoA by lysophosphatidic acid stimulation or by transfection of constitutively active RhoA (V14RhoA) or by the active RhoA-specific GEF onco-Lbc (Zheng *et al*., 1995) was capable of overriding these effects of SB-431542 (Supplementary Figure 3). Taken together, our data indicate that ALK5 kinase activity directly controls cell morphology and actin dynamics by regulation of basal RhoA activity.

ALK5 regulates basal RhoA activity independently of Smad2, Smad3 and Smad4

The rapid effect of SB-431542 treatment on RhoA–ROCK signalling indicated that ALK5-mediated regulation of this pathway was likely to be independent of the canonical Smad pathway. To formally address this issue, we measured the effects of SB-431542 on RhoA–ROCK signalling in a panel of Smad knockout MEFs. Immunofluorescence analysis revealed that 15 min treatment of wild-type (Wt), Smad2 knockout (Smad2−/−), Smad3 knockout (Smad3−/−) and Smad4 knockout (Smad4−/−) MEFs with 10 μM SB-431542 resulted in a comparable decrease in PO_4-MLC levels similar to those observed after treatment with Y27632 (Figure 2a). Western blotting analysis confirmed this result and indicated that SB-431542 treatment resulted in a dose-dependent decrease in the steady-state level of PO_4-MLC, irrespective of the Smad expression status of the MEFs (Figure 2b).

Autocrine TGF-β regulates basal RhoA activity in ^{V12}HaRas-transformed human diploid fibroblasts

Our data so far indicate that ALK5 regulates basal RhoA activity in rodent fibroblasts in a Smad-independent manner. We next aimed to determine whether this pathway is operative in human fibroblasts. We first tested the effects of SB-431542 on hTERT-immortalized human diploid fibroblasts (Tif-puro) and their ^{V12}HaRas-transduced counterparts (Tif-ras) (Ozanne *et al*., 2006). SB-431542 treatment (10 μM) of Tif-ras cells for 15 min disrupted actin stress fibres, lowered PO_4-MLC levels and abrogated focal adhesions (Figures 3a–c). Similarly, SB-431542 treatment resulted in a dose-dependent decrease in steady-state levels of RhoA-GTP (Figure 3d). Intriguingly, we observed that the Tif-Puro cells were resistant to SB-431542-mediated inhibition of RhoA signalling even though they have a functional ROCK-MLC2 pathway as revealed by Y27632 treatment (Figures 3a–d). This raised the possibility that the Tif-puro cells were incapable of responding to either the inhibitor or TGF-β. Western blotting analysis indicated that untreated Tif-puro and Tif-ras cells exhibited a comparable basal level of PO_4-Smad2 that was similarly susceptible to SB-431542-mediated inhibition (Figure 3e). Furthermore, both cell lines responded to exogenous TGF-β treatment, and SB-431542 inhibited phosphorylation of Smad2 with comparable efficiency. Similarly, SB-431542 treatment inhibited basal PO_4-Smad1 levels in Tif-puro and Tif-ras cells and blocked TGF-β-mediated stimulation of PO_4-Smad1 in Tif-ras cells (Supplementary Figure 4). Importantly, SB-431542 treatment did not block BMP4-mediated stimulation of PO_4-Smad1 in Tif-ras and Tif-puro cells (compare lanes 2 with 6 and lanes 8 with 12, Supplementary Figure 4), confirming the responsiveness to and specificity of SB-431542 in these cells.

Having determined that ALK5 regulates RhoA–ROCK signalling, we next aimed to determine whether this was ligand-dependent. ELISA analysis showed that NIH3T3, REF52, Wt MEFs, ALK5 null MEFS, Tif-puro and Tif-ras cells secrete latent TGF-β1 (Supplementary Table 1), which is consistent with the hypothesis that autocrine TGF-β signalling regulates RhoA through ALK5. To test this hypothesis, we transiently knocked down TGF-β1 production in the Tif-puro and Tif-ras cells. We efficiently knocked down TGF-β1 production by 80–90% with two different TGF-β1 siRNAs in both cell lines (Figure 4a). In agreement with our inhibitor studies, we found that knockdown of TGF-β1 decreased the steady-state level of PO_4-MLC2 in the Tif-ras cells but not in the Tif-puro cell line (Figure 4b). These experiments show that RhoA activity in normal human diploid fibroblasts is not regulated by TGF-β signalling but upon oncogenic transformation by ^{V12}Ha-Ras, RhoA activity becomes rewired through an autocrine TGF-β-ALK5 signalling pathway.

TGF-β-mediated regulation of basal RhoA activity is required for efficient oncogene-mediated transformation

Elevated RhoA activity is required for efficient transformation of mouse fibroblasts by ^{V12}HaRas and is selected for by an unknown mechanism during the transformation process (Qiu *et al*., 1995; Olson *et al*., 1998; Sahai *et al*., 2001). Owing to this intimate relationship of Ras and RhoA in oncogenic transformation and our data indicating that autocrine TGF-β regulates RhoA activity in ^{V12}HaRas-transduced human fibroblasts, we investigated the hypothesis that Ras-mediated transformation may rely on TGF-β signalling. We performed oncogene cooperation transformation assays in primary rat embryo fibroblasts (REFs) using combinations of activated ^{V12}HaRas and either mutant p53 or adenovirus E1a. Transfection of these cells with activated ^{V12}HaRas alone resulted in a low transformation rate, which was greatly increased by co-expression of mutant p53 or E1a (Figure 5a). Co-treatment with 10 μM SB-431542 reduced the number of transformed colonies by 75% in the Ras and mutant p53 transfectants and by 90% in the Ras and E1A transfectants (Figure 5a). SB-431542 treatment did not reduce the proliferation rate of primary REFs (Supplementary Figure 5a), indicating that the oncogene-mediated transformation of these cells specifically requires ALK5 kinase activity.

We obtained similar results using the well-characterized NIH3T3 focus formation transformation assay. In this assay, transfection of ^{V12}HaRas alone results in efficient transformation and this has been shown to require RhoA activity (Sahai *et al*., 1999). Activated ^{V12}HaRas, ^{D12}HaRas and V-Raf readily transformed NIH3T3 cells but the appearance of transformed colonies was reduced by approximately 50–60% with 10 μM SB-431542 treatment (Figure 5b). Similarly, oncogenic transformation of NIH3T3 cells by ^{V600E}BRAF was also inhibited by 10 μM SB-431542 treatment (Figure 5c). Further we confirmed that this effect was not due to SB-431542 regulating the basal proliferation rates of these cells (Supplementary Figure 5b).

Sustained activation of the RhoA–ROCK pathway is also required to support the ability of Ras or Rho-GEF-

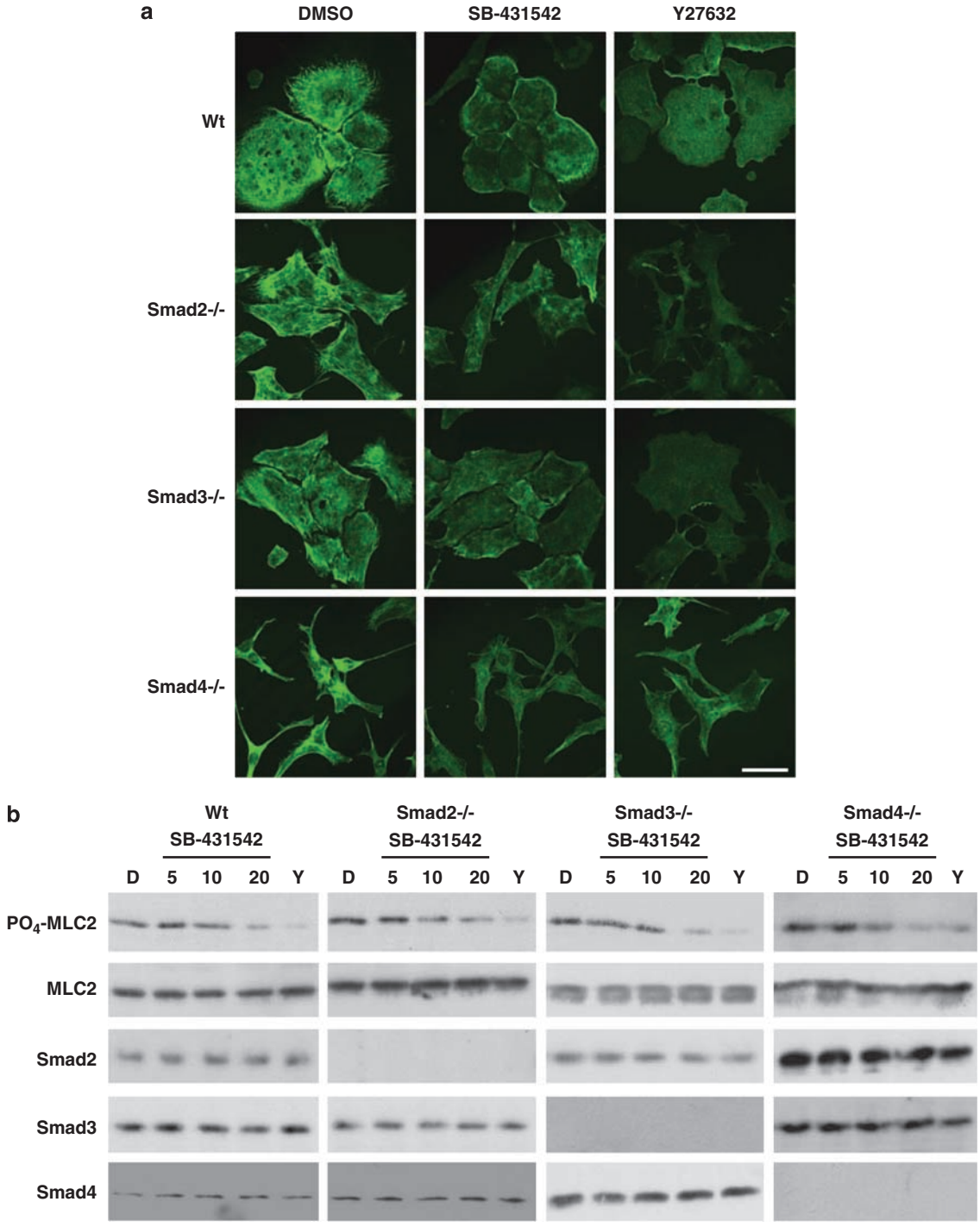

Figure 2 SB-431542-mediated inhibition of RhoA–ROCK signalling is Smad independent. (**a**) Serum-starved wild-type (Wt) and Smad2, Smad3 and Smad4 knockout MEFs were treated with 10 μM SB-431542 or 10 μM Y27632 for 15 min prior to fixation and analysis by immunofluorescence microscopy using PO_4-MLC antibodies. Scale bar indicates 20 μm. (**b**) Knockout MEFs were treated as in panel a with the indicated concentrations of inhibitors. PO_4-MLC2, total MLC2 and Smad2, Smad3 and Smad4 levels were analysed by western blotting.

transformed cells to grow in an anchorage-independent manner (Sahai *et al*., 1999). Treatment of NIH3T3 cells stably transformed with ^{V12}HaRas (NIH3T3-ras) with 10 μM SB-431542 reduced the number of colonies formed in soft agar by 50% (Figure 5d), but did not significantly affect the proliferation of these cells when cultured on plastic (Supplementary Figure 5c), indicating ALK5 activity supports anchorage-independent growth.

To corroborate our inhibitor assays, we further investigated the contribution of ALK5 and TGF-βRII to transformation by co-transfecting NIH3T3 cells with ^{V12}HaRas and a kinase dead mutant of ALK5 (ALK5KD) and a dominant-negative TGF-βRII

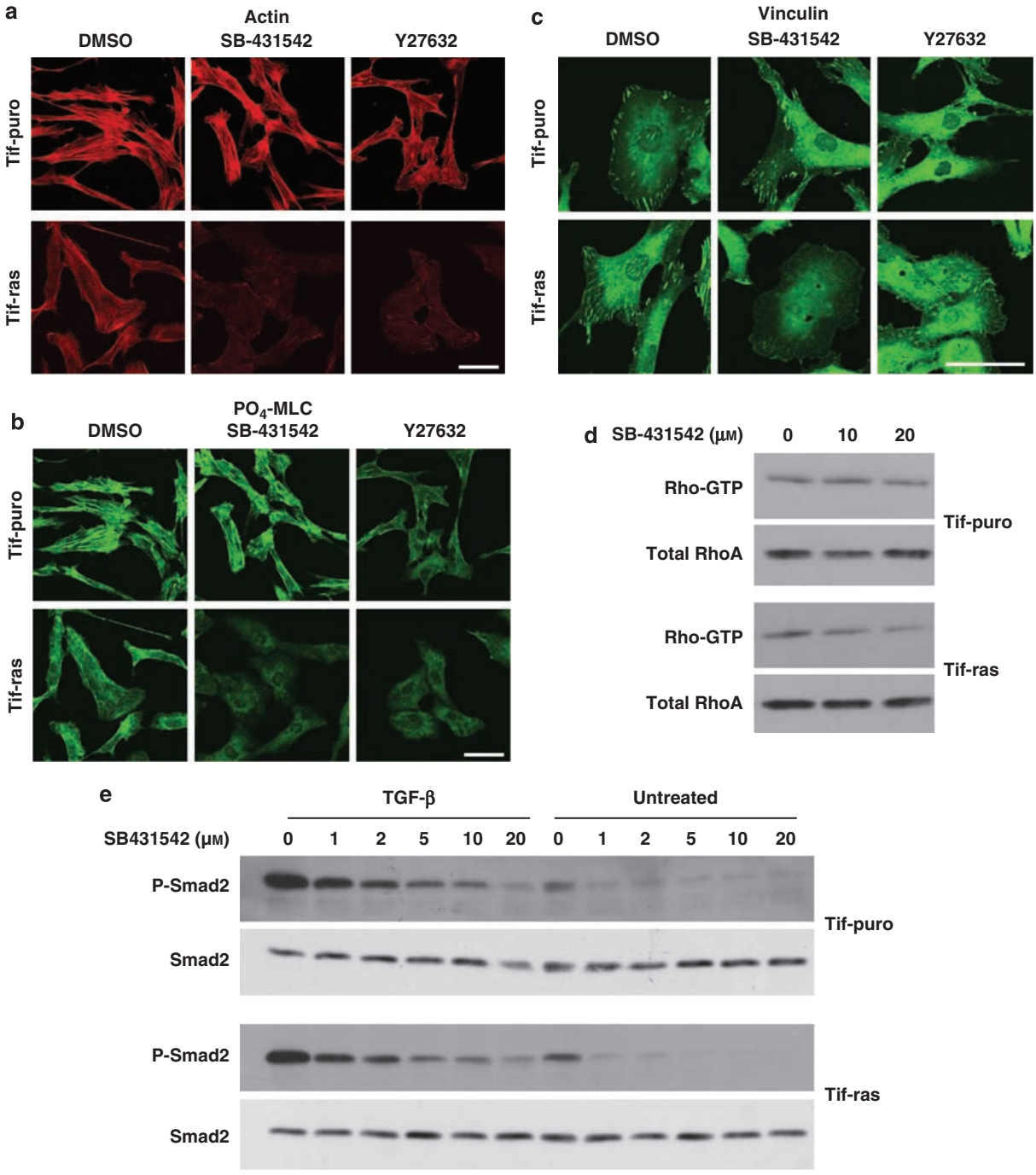

Figure 3 ALK5 regulates RhoA signalling in ^{V12}HaRas-transformed human diploid fibroblasts**.** Serum-starved hTERT-immortalized control (Tif-puro) and ^{V12}HaRas-transformed human diploid fibroblasts (Tif-ras) were treated for 15 min with 10 μM SB-431542, 10 μM Y27632 or DMSO and were fixed and stained for actin (**a**), PO_4-MLC2 (**b**) and vinculin (**c**) and analysed as in Figure 1. Scale bars are 20 μm. (**d**) Serum-starved cells were treated with SB-431542 for 15 min and RhoA-GTP and total RhoA levels were analysed as in Figure 1. (**e**) Cells were treated with the indicated concentrations of SB-431542 for 15 min prior to TGF-β treatment for 1 h (TGF-β) or not (untreated). Levels of PO_4-Smad2 and Smad2 were determined by western blotting.

(DN-RII). In accordance with our inhibitor studies, we found that interfering with TGF-β signalling reduced transformation efficiency to a comparable rate as co-transfection of a dominant-negative RhoA (DN-RhoA), whereas further stimulation of RhoA by transfection of the constitutively active RhoA-specific GEF, onco-Lbc, had no effect (Figure 6a). We next assessed whether inhibition of RhoA–ROCK and TGF-β signalling together further decreased transformation efficiency. Y27632 treatment reduced transformation by ^{V12}HaRas to a similar extent as SB-431542 treatment and combined treatment did not result in a further decrease in transformation, indicating that inhibition of ALK5 or ROCK is equivalent in this assay (Figure 6b). This

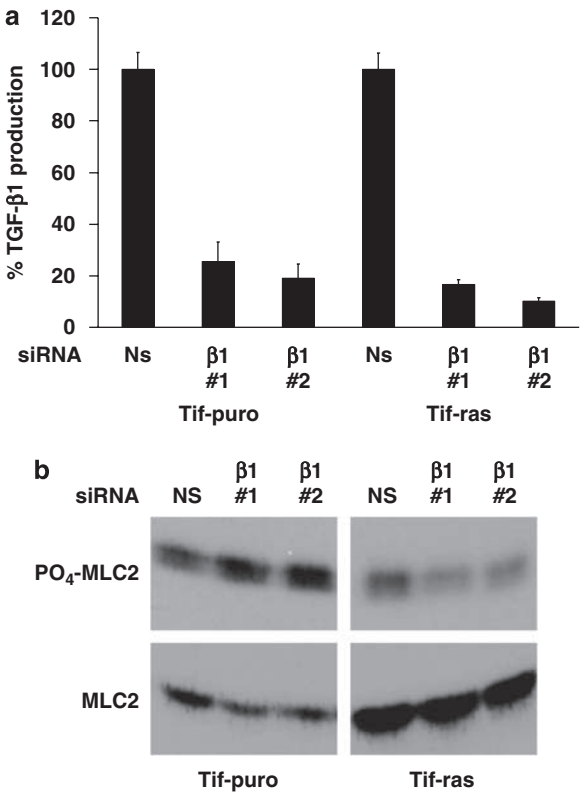

Figure 4 Autocrine TGF-β regulates RhoA–ROCK signalling in ^{V12}HaRas-transformed human diploid fibroblasts. (**a**) Cells were transfected with control (non-silencing Ns) or with two independent TGF-β1 siRNAs (β1 no. 1, β1 no. 2). Culture media were replaced with 0.1%FCS/DMEM 24 h after transfection, removed after a further 16 h and the total amount of TGF-β1 present determined by ELISA. TGF-β1 levels are normalized to cell number and represented as the percentage of a mock-transfected control for each cell type. Means ± s.d. are shown ($n = 3$). (**b**) Cells were transfected as in panel a and then analysed for PO_4-MLC2 and MLC2 levels by western blotting.

observation suggests that ALK5 and RhoA–ROCK operate in the same pathway during transformation. We tested this hypothesis further by simultaneously stimulating RhoA activity and inhibiting ALK5 activity during transformation. Co-transfection of constitutively activated RhoA-specific GEFs, Δ558LARG and onco-Lbc, or the activated mutant ^{V14}RhoA with ^{V12}HaRas was sufficient to override SB-431542-mediated inhibition of transformation (Figure 6c), indicating that ALK5 activity contributes to transformation solely by activating RhoA.

Discussion

TGF-β signals through a heterooligomeric complex of TβRII and ALK5 to activate a myriad of downstream signalling pathways that ultimately culminate in a change of gene expression and biological response. TGF-β signalling invariably involves activation of the Smads and may also include activation of the ERK–MAPK, PI3K, RhoA, Cdc42, AKT and p38MAPK pathways in a context, cell-type and dose-dependent manner. Integration and cross-talk between these and other signalling networks operative in the target cell provide a mechanism of fine-tuning cellular response under both normal growth control and pathobiological situations including cancer. TGF-β can act as both a potent tumour suppressor and a potent promoter of metastasis in a context-dependent manner, but a molecular understanding of how and when TGF-β acts in a pro-oncogenic manner is limited (Pardali and Moustakas, 2007). Recent data indicate that this may involve Smad activation, and in the case of glioma (Bruna *et al*., 2007) and breast cancer (Kang *et al*., 2005), high Smad activity correlates with poor prognosis. It has earlier been proposed that TGF-β-mediated regulation of non-Smad pathways may also contribute to the pro-oncogenic actions of TGF-β (Wakefield and Roberts, 2002; Roberts and Wakefield, 2003). Here, to the best of our knowledge, we provide the first direct evidence in support of this hypothesis. Studies of others have indicated that TGF-β can activate RhoA signalling during epithelial-to-mesenchymal transition, and that inhibition of RhoA signalling blocks TGF-β-induced epithelial-to-mesenchymal transition (Bhowmick *et al*., 2001; Vardouli *et al*., 2005). TGF-β-mediated epithelial-to-mesenchymal transition is likely to contribute to the tumour-promoting activities of TGF-β (Pardali and Moustakas, 2007), but it has yet to be indicated if this requires Smad-independent activation of this process. Here we show that ALK5 activity maintains GTP-RhoA levels and that this is required for oncogene-mediated transformation and identifies a new Smad-independent pro-oncogenic activity of TGF-β.

Our studies in serum-starved rodent fibroblasts revealed that treatment with the ALK5 kinase inhibitor SB-431542 decreased basal RhoA-GTP levels. Consistent with these findings, ALK5 inhibition lead to a rapid decrease in PO_4-MLC levels, actin stress fibre formation and focal adhesions, which are processes regulated by RhoA–ROCK signalling (Jaffe and Hall, 2005). The rapid kinetics of TGF-β-mediated activation of RhoA and SB-431542-mediated inhibition of basal GTP-RhoA levels suggested that these events were likely to be independent of the canonical Smad pathway. This was confirmed in studies employing knockout MEFs, which revealed that ALK5 regulates RhoA–ROCK signalling independently of Smad2, Smad3 and Smad4.

In contrast to our studies in rodent fibroblasts, we observed that in hTERT-immortalized human diploid fibroblasts, this connection was only operative upon transformation by ^{V12}HaRas and was mediated by autocrine TGF-β1. This finding coupled with earlier observations, which have found that efficient transformation by ^{V12}HaRas requires high RhoA-GTP levels (Olson *et al*., 1998; Sahai *et al*., 2001), promoted us to investigate the role of TGF-β signalling in rodent cell transformation assays. Strikingly, we discovered that ALK5-mediated activation of RhoA is required for efficient transformation mediated by ^{V12}HaRas and ^{V600E}BRAF. These data suggest that the hitherto unknown selection mechanism that takes place during

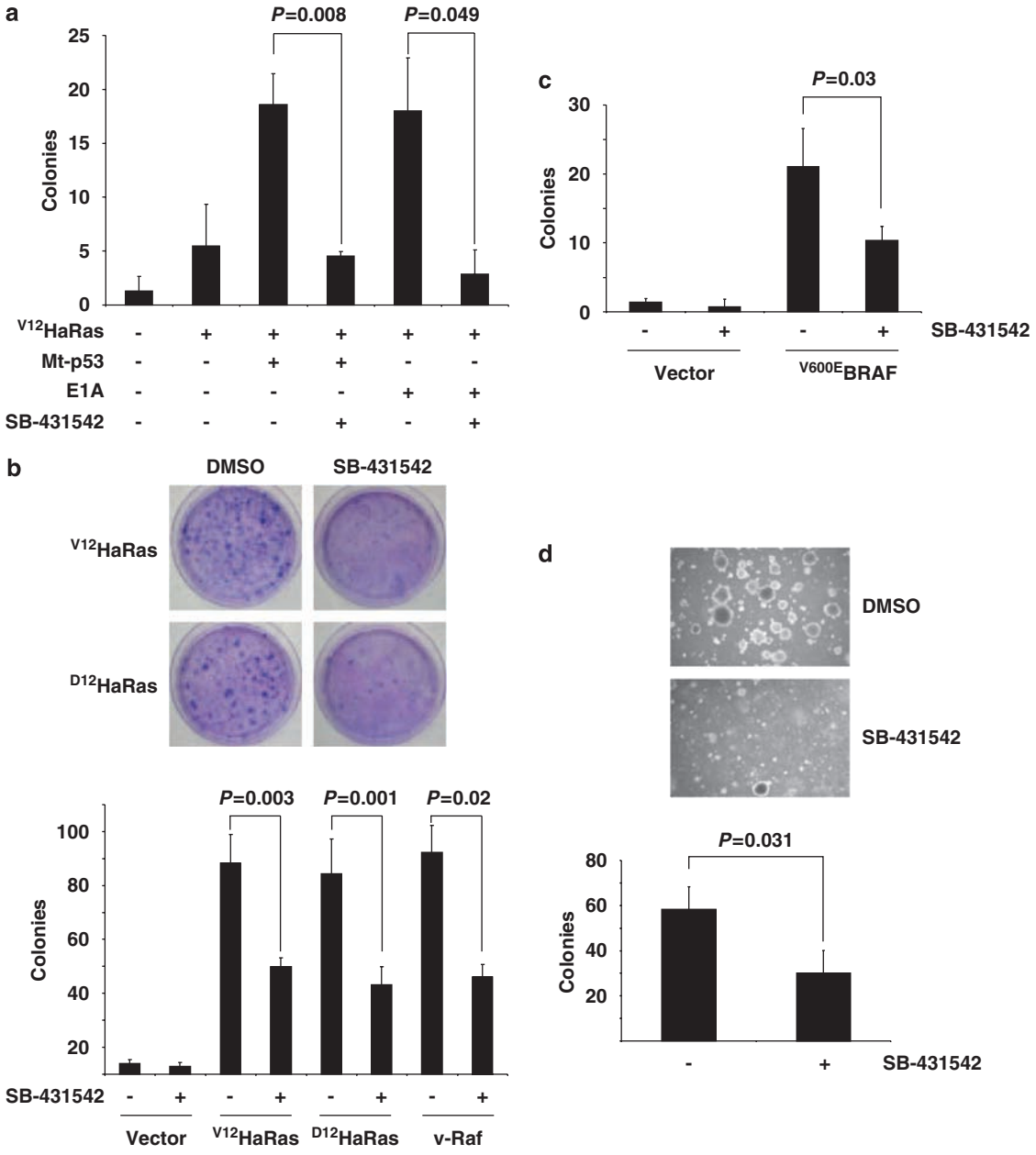

Figure 5 ALK5 activity is required for efficient oncogene-induced transformation. (**a**) Primary REFs were transfected with plasmids expressing the indicated proteins, and transformation assays were performed with or without 10 μM SB-431542 present in the selection media. Morphologically transformed colonies were counted under microscopic examination. Data shown are mean ± s.e.m. ($n = 3$). (**b** and **c**) NIH3T3 focus formation assays. Cells were transfected with the indicated plasmids and maintained in 5% serum for 14 days with or without 10 μM SB-431542 or DMSO before being fixation and staining. Photographs of representative stained dishes and quantification of foci are shown. Results represent the mean ± s.e.m. (**b**, $n = 7$ vector, ^{V12}HaRas; $n = 3$ v-Raf; (**c**) $n = 3$). (**d**) NIH3T3-Ras cells were cultured in soft agar in the presence of DMSO or 10 μM SB-431542 for 21 days prior to photography and quantification of colonies. Results represent the mean ± s.e.m. ($n = 3$) and *P*-values are shown.

^{V12}HaRas transformation for high RhoA activity (Olson *et al*., 1998; Sahai *et al*., 2001) is driven by autocrine TGF-β. Earlier studies have indicated that high RhoA activity acts to suppress the expression of the cyclin-dependent kinase inhibitor p21, which is induced by oncogenic activation of the MAPK pathway to allow proliferation of the transformed cells (Olson *et al*., 1998). We are currently investigating the possibility that autocrine TGF-β-mediated activation of RhoA contributes to the transformation process by suppressing p21 expression.

Our data presented here indicate that autocrine TGF-β regulates basal RhoA activity in rodent and ^{V12}HaRas-transformed human fibroblasts in an ALK5 kinase-dependent manner. The finding that this pathway is not operative in hTERT-immortalized fibroblasts indicates that oncogenic transformation rewires RhoA signalling through ALK5 by an unknown mechanism. The activity

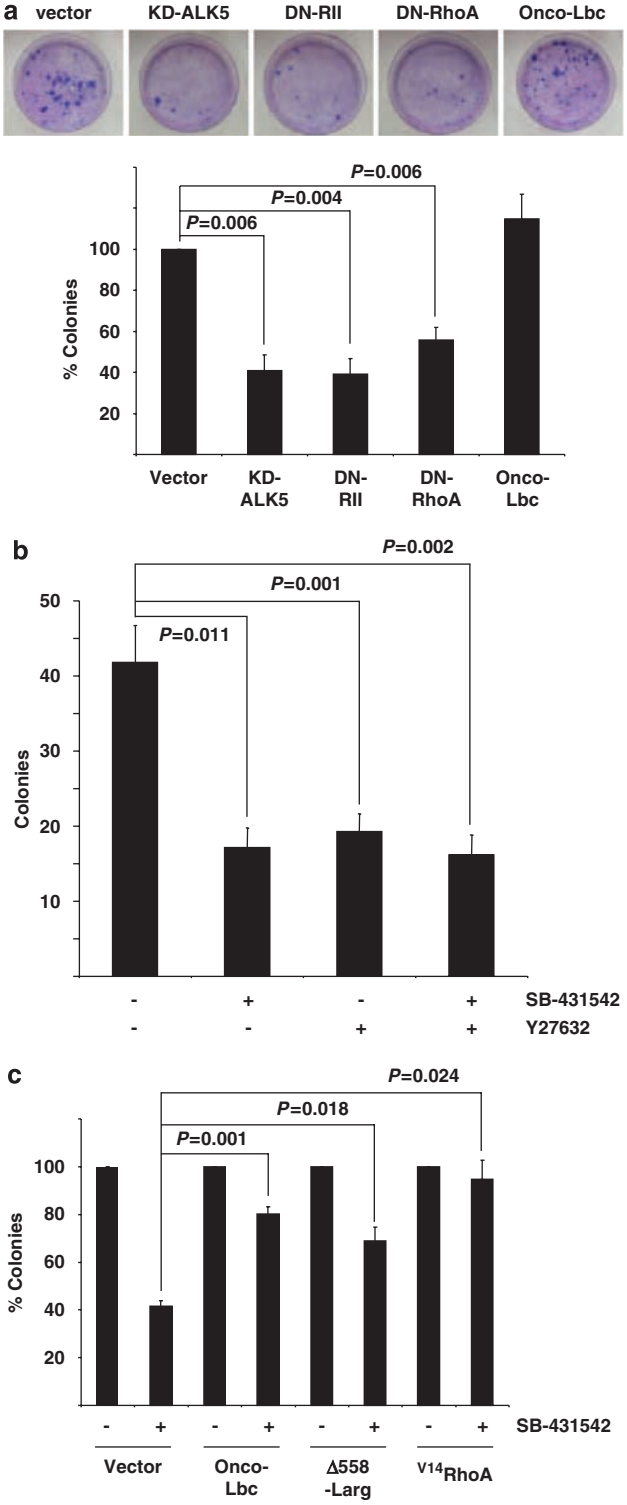

Figure 6 TGF-β receptor-mediated RhoA activation is required for oncogene transformation. (**a**–**c**) NIH3T3 focus formation assays of cells co-transfected with ^{V12}HaRas and the indicated expression plasmids were performed as described in Figure 5. (**a**) Dominant-negative (DN) TGF-β receptors and DN-RhoA inhibit ^{V12}HaRas transformation. Photographs of representative stained dishes and quantification of foci are shown. (**b**) Cells were treated with 10 μM SB-431542 and 10 μM Y27632 as indicated. (**c**) Stimulation of RhoA signalling by co-transfection of activated RhoA-GEFs (Onco-Lbc, Δ558-Larg) or activated RhoA overrides SB-431542-mediated inhibition of transformation. Results represent the mean ± s.e.m. ($n \geqslant 4$) and *P*-values are shown.

of RhoA is controlled by the coordinated actions of GEFs and GAPs. It is conceivable that ALK5 either directly phosphorylates a GEF to activate its RhoA-specific GTP exchange activity or phosphorylates a GAP to inhibit its GTPase-stimulating activity, and it is also possible that oncogenic activation of the Ras–MAPK pathway regulates expression of one or more of these factors.

Our findings that anchorage-independent growth of NIH3T3-ras cells is inhibited by SB-431542 treatment as well as by *de novo* transformation of rodent fibroblasts by ^{V12}HaRas and ^{V600E}BRAF indicates that the maintenance of the transformed phenotype as well as the initiation of transformation requires ALK5 kinase activity. These observations raise the exciting possibility that human tumours driven by mutation of these oncogenes may also require ALK5 activity for sustained proliferation.

Materials and methods

Antibodies and reagents

Antibodies against the following proteins were used: actin, vinculin, MLC2, FLAG M2 (Sigma, Poole, UK); phospho-MLC2 (Ser-19) and PO_4-Smad2 (Cell Signalling Technology, Beverly, MA, USA); RhoA, MLC2 and p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); TGF-β1 (R&D Systems, Abingdon, UK) and Smad2/3 (BD Biosciences, Oxford, UK). SB-431542 (Tocris, Bristol, UK), Y27632 (Calbiochem, San Diego, CA, USA), lysophosphatidic acid (Sigma) and recombinant human TGF-β1 (Peprotech, London, UK) were used at final concentrations as appropriate.

Cell culture, plasmids and transfections

Primary Fisher REFs (Biowhittaker, Wokingham, UK), NIH3T3 cells, the rat embryo fibroblast cell line REF52, hTERT-immortalized fibroblasts (Tif-puro, Tif-ras) and Smad knockout MEFs were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine and 100 U/ml penicillin and streptomycin. ALK5 wild-type and null MEFs were grown in high-glucose DMEM supplemented with 7% FCS and 100 μM non-essential amino acids. Cells were serum-starved in media containing 0.1% FCS overnight where indicated, and all experiments were performed using subconfluent cells. We are grateful for provision of the following plasmids: pRK5myc-^{V14}RhoA (A Hall), onco-Lbc (M Olson), EJ6.6 and mt^{R248W}p53 (T Crook). DNA plasmids were introduced into REF52 cells using Polyfect (Qiagen, Crawley, UK) and into NIH3T3 cells using Lipofectamine (Invitrogen, Inchinnan, UK). Transient transfection assays were performed as described earlier (Inman *et al*., 2002) TGF-β1 and Allstar control siRNA oligonucleotides (Qiagen) were introduced into human fibroblasts using Dharmafect3 reagent (Thermo Scientific, Runcorn, UK) and were used at a final concentration of 50 nM.

RhoA-GTP assays, western blotting and immunofluorescence

Levels of active GTP-RhoA were determined by incubating lysates with GST-rhotekin-RBD bound to glutathione-coupled agarose beads as described (Goulimari *et al*., 2005). Western blotting was performed as described earlier (Inman *et al*., 2002) except for analysis of PO_4-MLC2 and MLC2 where cell lysates were made directly in 4 × SDS Laemmelli sample

buffer. For immunofluorescence, cells were fixed with 5% paraformaldehyde, washed with phosphate-buffered saline/100 mM glycine and permeabilized with phosphate-buffered saline/0.1% saponin/20 mM glycine. After blocking with phosphate-buffered saline/0.1%, saponin/10% FCS cells were incubated with appropriate primary antibodies and were visualized with species-specific Alexa Fluor 488-coupled secondary antibodies (Invitrogen). Actin filaments were visualized with tetramethylrhodamine α-isothiocyanate (TRITC)-phalloidin (Sigma).

Microscopy and image processing
Images of fixed cells were obtained by confocal microscope (Leica SP2) equipped with HCX PL APO ×40, 1.25 and HCX PL APO ×63, 1.4–0.6 oil immersion lenses. Images of stained plates from transformation assays were obtained using a Li-cor Odyssey infrared imager and software.

TGF-β1 ELISA
Cells were cultured overnight in media containing 0.1% FCS. The following day the medium was removed and TGF-β1 levels were determined by ELISA. This assay used monoclonal TGF-β antibody as a capture antibody and biotinylated polyclonal TGF-β1 antibody (R&D Systems) as a detection antibody. To measure total TGF-β1 present in tissue culture media, biologically latent TGF-β1 was activated by acid treatment. Recombinant TGF-β1 was used as standard at 31.2–2000 pg/ml and background TGF-β1 present in 0.1% FCS media was subtracted from all values. After media removal, cells were trypsinized and counted to allow the calculation of the amount of TGF-β1 produced per cell per hour.

Transformation assays
Transformation of primary REFs was performed using calcium phosphate-mediated transfection as described earlier (Crook *et al*., 1994). After transfection, transformed colonies were selected for growth in 500 μg/ml G418 (Invitrogen) for 3–4 weeks before being fixed and stained in Giemsa (modified stain; Sigma). NIH3T3 transformation assays were carried out as described (Sahai *et al*., 1998).

Soft agar assays
Six-well dishes were coated with DMEM (10% FBS, L-glutamine, penicillin/streptomycin) supplemented with 0.9% agarose. Cells were seeded on top at a density of 2×10^4 cells/well in DMEM supplemented with 0.45% agarose, ± TGF-β or SB-431542 or carrier controls at the appropriate concentration and were incubated at 37 °C, 10% CO_2. A volume of 300 μl of DMEM ± additives was added to each well twice weekly. Colonies were counted 2–4 weeks post-seeding. Colonies >80 μm in diameter were scored and the indicated number of 5250 μM^2 fields/well were analysed using a Olympus CKX41 microscope fitted with a ×4 objective and an eyepiece graticule. Images were captured using a Qimaging Retiga EXi digital camera and QCapture Pro software (QImaging corporation).

Statistical analysis
Two-tailed Student's *t*-tests were used to compare two groups and *P*-values are shown where appropriate. The number of biological replicates (*n*) is indicated in each case.

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