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ORIGINAL ARTICLE

Loss of human Scribble cooperates with H-Ras to promote cell invasion through deregulation of MAPK signalling

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Activating mutations in genes of the Ras-mitogen-activated protein kinase (MAPK) pathway occur in approximately 30% of all human cancers; however, mutation of Ras alone is rarely sufficient to induce tumour development. Scribble is a polarity regulator recently isolated from a *Drosophila* screen for events that cooperate with Ras mutation to promote tumour progression and cell invasion. In mammals, Scribble regulates directed cell migration and wound healing *in vivo*; however, no role has been identified for mammalian Scribble in oncogenic transformation. Here we show that in human epithelial cells expressing oncogenic Ras or Raf, loss of Scribble promotes invasion of cells through extracellular matrix in an organotypic culture system. Further, we show that the mechanism by which this occurs is in the regulation of MAPK signalling by Scribble. The suppression of MAPK signalling is a highly conserved function of Scribble as it also prevents Raf-mediated defects in *Drosophila* wing development. Our data identify Scribble as an important mediator of MAPK signalling and provide a molecular basis for the observation that Scribble expression is decreased in many invasive human cancers.
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Introduction

The development of tumours requires a number of genetic and epigenetic alterations, including both the loss of tumour suppressive mechanisms and the acquisition of tumour promoting, or oncogenic changes. Activating mutations in the Ras-Raf-mitogen-activated protein kinase (MAPK) pathway are seen in nearly one-third of all human cancers (Dhillon *et al*., 2007); however, mutation of Ras or Raf alone is rarely sufficient to promote tumourigenesis, rather it leads to cell senescence (Serrano *et al*., 1997; Michaloglou *et al*., 2005; Sarkisian *et al*., 2007). Scribble is a polarity regulator (Bilder and Perrimon, 2000) that was recently identified in a *Drosophila* screen for mutations that cooperate with activated Ras to promote tumour progression (Pagliarini and Xu, 2003). Loss of Scribble or the related polarity regulators, Dlg and Lgl, is sufficient to induce tumourigenic growth and promote spontaneous invasion and metastasis in combination with activated oncogenes Ras, Raf or Notch (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). Moreover, loss of these polarity regulators alone in *Drosophila* results in neoplastic tissue overgrowth (Bilder *et al*., 2000) and *lgl* mutant cells form metastatic tumours in *Drosophila* transplant models (Woodhouse *et al*., 1998). In fact, loss of *dlg* or *lgl* in the early *Drosophila* embryo causes inappropriate invasion of follicular epithelial cells in the absence of secondary changes (Goode and Perrimon, 1997; Abdelilah-Seyfried *et al*., 2003). While this data suggest that Scribble, Dlg and Lgl negatively regulate cell movement, recent studies using *Drosophila*, rodent and human cells indicate that in some circumstances these proteins are required for normal cell migration (reviewed in (Dow and Humbert, 2007)). Thus it remains unclear whether the role for Scribble in restricting cell invasion is unique to certain *Drosophila* cell types or indicates a context-dependent role for Scribble in the regulation of cell motility (Humbert *et al*., 2006).

Scribble has also been linked to the development of mammalian tumours *in vivo*. Scribble and Dlg are both targeted for degradation by the E6 oncoprotein from 'high-risk' human papilloma viruses HPV16 and HPV18 (Gardiol *et al*., 1999; Nakagawa and Huibregtse, 2000), causally linked to greater than 90% of cervical cancers. Consistent with this, studies have now demonstrated that Scribble expression is decreased in tumours associated with HPV infection (Massimi *et al*., 2004; Nakagawa *et al*., 2004). The degradation of PDZ-containing proteins such as Scribble is essential for E6-mediated cell transformation because mutants that lack this motif, but can still inactivate p53, cannot induce hyperplasia or cell transformation such as wild-type E6 (Kiyono *et al*., 1997; Nguyen *et al*., 2003).

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In fact, Scribble overexpression can suppress the transforming potential of HPV E6/E7 proteins in rodent epithelial cells (Thomas *et al*., 2005). High-risk HPV strains also synergize with activation of Ras or the epidermal growth factor receptor (EGFR) to promote cell transformation *in vitro* (Storey and Banks, 1993; Woodworth *et al*., 2000) and tumourigenesis *in vivo* (Schreiber *et al*., 2004). These data, combined with the observed loss of Scribble in cancers such as colon and breast (Navarro *et al*., 2005; Gardiol *et al*., 2006) prompted us to directly test whether loss of Scribble cooperates with activation of Ras in mammalian oncogenesis. Using organotypic 3D cultures, we show that loss of mammalian Scribble cooperates with H-Ras^{V12} to induce invasion of mammary epithelial cells and that the major mechanism by which this cooperation occurs is through regulation of MAPK signalling by Scribble. These data reveal a novel function for Scribble in modulating MAPK-signalling and demonstrate for the first time the highly conserved role for Scribble as an invasion suppressor.

Results

Loss of Scribble promotes Ras-dependent cell invasion

To examine whether loss of human Scribble would cooperate with activation of Ras signalling to promote cell invasion as in *Drosophila*, we concurrently induced both loss of Scribble (via small-hairpin RNA, shRNA) and activation of Ras signalling in non-transformed, human MCF10A breast epithelial cells. Polyclonal MCF10A populations expressing Scribble-targeted shRNA (Scribble^{KD}) or H-Ras^{V12}-IRES-GFP (referred to as Ras^{V12}) showed approximately fivefold reduction in Scribble protein and/or 5–10 overexpression of Ras^{V12} (Figure 1a). Expression of a scrambled shRNA (pRS) had no effect on the level of Scribble expression. At this moderate level of expression, Ras^{V12} caused a disruption to the cobblestone morphology of MCF10A cells in 2D culture and they appeared spindle-shaped without the cohesive epithelial junctions of control cells (Supplementary Figure 1). Consistent with a disruption to normal junction formation, Ras^{V12} expressing cells showed a significantly reduced level of the adherens junction (AJ) protein E-cadherin (Figure 1a), which correlated with a reduction in *E-cadherin* mRNA (Figure 4f). Depletion of Scribble had no obvious effects on the morphology of MCF10A cells expressing Ras^{V12} in 2D culture (Supplementary Figure 1).

To determine the effect of Ras^{V12} expression and loss of Scribble on epithelial architecture, cells were cultured in Matrigel reconstituted basement membrane. Under these conditions, MCF10A cells form hollow, growth-arrested acini after 10–12 days in culture (Debnath *et al*., 2003; Dow *et al*., 2007). Constitutive expression of Ras^{V12} resulted in acini that looked similar to vector and Scribble^{KD} control acini at low magnification (Figure 1b); however, these structures were not polarized, had no discernable lumen and did not undergo normal cell-cycle exit such as control acini (Supplementary Figure 1B and C). Similarly, Scribble^{KD}-Ras^{V12} acini were not polarized and remained Ki67 positive (Supplementary Figure 1B, C); however, they also had cellular protrusions that projected out from the acinar structure (Figures 1b and c, arrows). Live imaging of cells between days 6 and 7 of culture revealed that Ras^{V12} expressing acini produce small, transient filopodial protrusions that retract within the acini over time (Figure 1d). In contrast, filopodial protrusions from Scribble^{KD}-Ras^{V12} acini extended further into the surrounding extracellular matrix (ECM) and over time the entire cell body moved through the ECM, invading away from the acinar structure (Figure 1d, Supplementary Movies 1 and 2). In some cases, invasion of Scribble^{KD}-Ras^{V12} cells resulted in long protrusive extensions made up of multiple cells (Figure 1e). To quantitate cell invasion, we calculated the percentage of acini exhibiting at least one invasive protrusion, visible at low magnification, at day 8 of 3D culture. At day 8, $21 \pm 3.6\%$ of Scribble^{KD}-Ras^{V12} acini showed invasion, compared with only $1.3 \pm 0.04\%$ for Ras^{V12} alone (Figure 1f). It is not clear why only a proportion of Scribble^{KD}-Ras^{V12} acini show protrusions, but a similar phenomenon is apparent in other studies using MCF10A cells (Seton-Rogers *et al*., 2004; Overholtzer *et al*., 2006). It is perhaps due to local changes in the surrounding ECM as published studies (Kim *et al*., 2004; Shin *et al*., 2005) and our own observations (not shown) indicate that matrix remodelling is essential for MCF10A invasion into Matrigel. Notably, we did not detect any change in the Ras-dependent upregulation of matrix metalloprotease 2 (data not shown). Importantly, expression of an RNA interference (RNAi) resistant version of Scribble (mouse Scribble) in the Scribble^{KD}-Ras^{V12} cells (Figure 1g), reverted the invasive behaviour in 3D culture (Figure 1h), confirming that it was loss of Scribble and not an off-target effect of shRNA expression responsible for the invasive phenotype. In addition, we have also observed the same invasive phenotype using *scribble* shRNA retroviral vectors designed to independent target sequences (data not shown).

MEK-ERK signalling is essential for invasion of Scribble-depleted Ras^{V12} cells

In *Drosophila*, loss of Scribble cooperates with activated Ras and Raf to promote invasion through ectopic activation of Jun N-terminal Kinase (JNK) signalling (Brumby and Richardson, 2003). To determine whether deregulation of MAPK signalling was responsible for the invasion of Scribble^{KD}-Ras^{V12} cells we examined the requirement for each of the canonical MAPK effector arms of Ras signalling: mitogen-activated protein kinase/extracellular-regulated kinase (MEK-ERK), JNK and p38 pathways. To do this, MCF10A 3D cultures were treated with selective inhibitors to MEK1/2 (PD98059), JNK1/2 (SP600125) and p38α/β (SB202190), all of which have been validated in MCF10A cells (Supplementary Figure 2; Joiakim *et al*., 2003; Seton-Rogers *et al*., 2004; Reginato *et al*.,

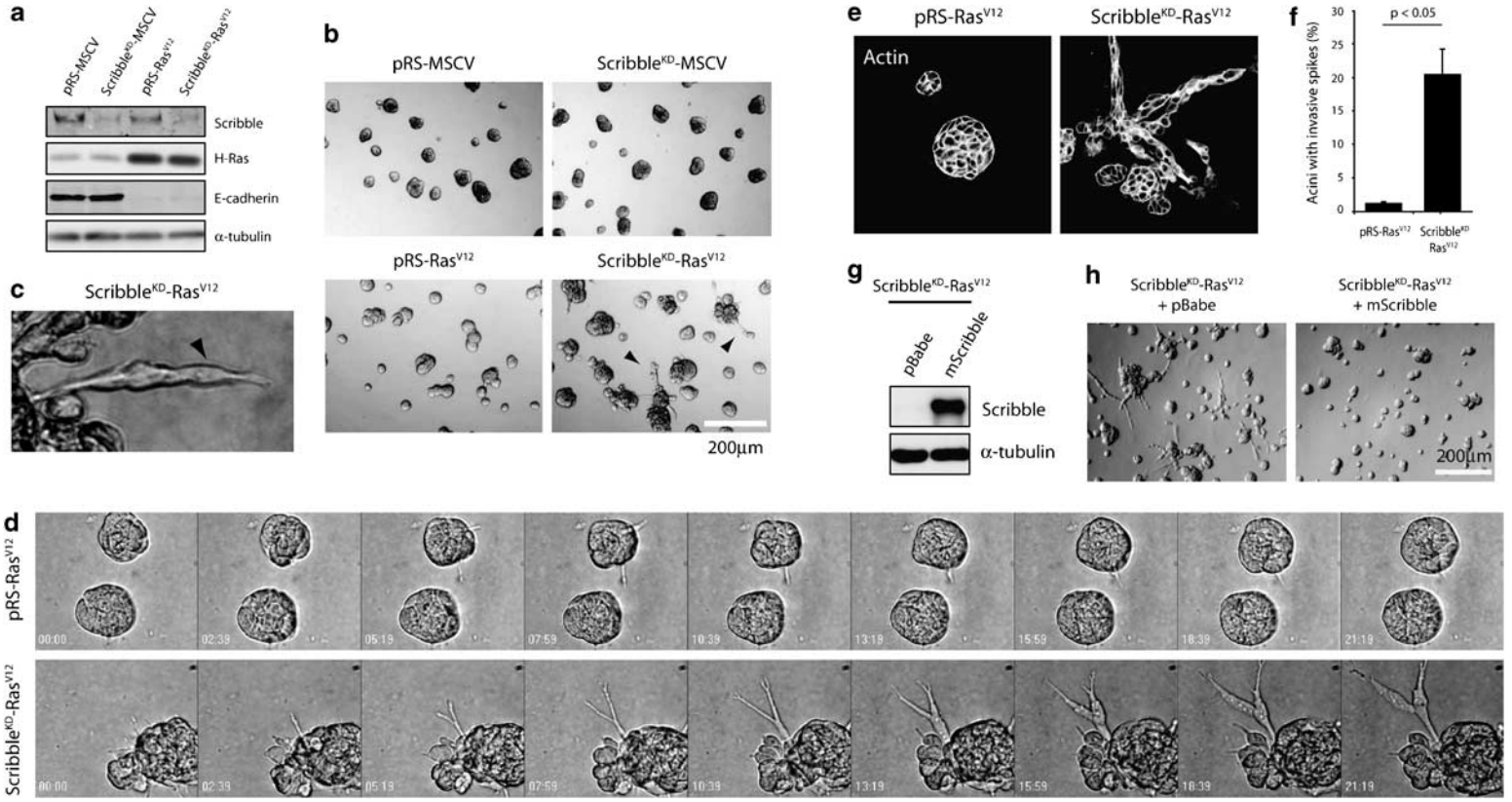


Figure 1 Loss of Scribble cooperates with Ras^{V12} to induce invasion in Matrigel culture. (**a**) Western blot of whole-cell lysates probed with antibodies indicated on the right side of each panel. (**b**) DIC images of MCF10A cell lines, as indicated, cultured in 100% growth factor-reduced (GFR) Matrigel for 8 days. (**c**) High magnification view of Scribble^{KD}-Ras^{V12} cell protrusion. (**d**) DIC time-lapse images of pRS-Ras^{V12} and Scribble^{KD}-Ras^{V12} acini cultured in GFR Matrigel. Time-lapse images acquired between days 6 and 7 of Matrigel culture. Elapsed time (hours:minutes) from the beginning of image acquisition shown in the lower left corner. (**e**) Confocal images of acini at day 8 of 3D culture labelled with Alexa-568-phalloidin to highlight F-actin. (**f**) Quantitation of the percentage of Ras^{V12} and Scribble^{KD}-Ras^{V12} acini with invasive protrusions. Graph represents the average of three independent experiments. Error bars are standard deviation of the mean ($n=3$, $P<0.05$, Student's *t*-test). Acini (50–200) were scored per cell line, per experiment. No increase in the percentage of invasive acini was observed after day 8 of culture in either cell population. (**g**) Western blot of whole-cell lysates showing expression of murine Scribble (mScribble) in Scribble^{KD}-Ras^{V12} cells. (**h**) Images of MCF10A cell lines indicated, cultured in 100% GFR Matrigel for 8 days.

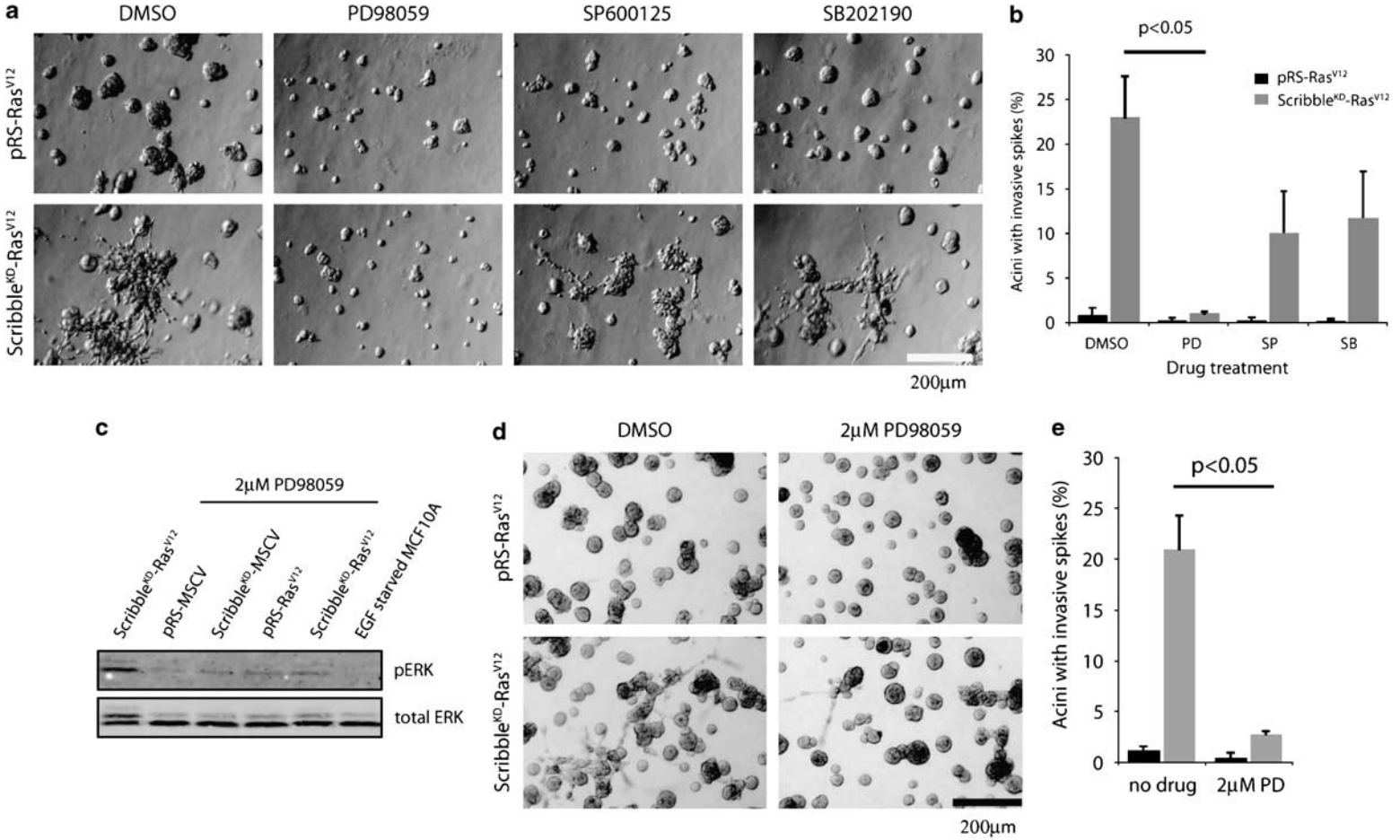


Figure 2 Invasion of Scribble^{KD}-Ras^{V12} cells is dependent on mitogen-activated protein kinase (MEK) but not Jun N-terminal Kinase (JNK) or p38. (**a**) Phase contrast images of pRS-Ras^{V12} and Scribble^{KD}-Ras^{V12} cells cultured in growth factor-reduced (GFR) Matrigel for 8 days in the presence of vehicle alone (dimethyl-sulphoxide, DMSO), MEK inhibitor: PD98059 (20 μM), JNK inhibitor: SP600125 (20 μM) or p38α/β inhibitor: SB202190 (20 μM). (**b**) Percentage of pRS-Ras^{V12} (black bars) and Scribble^{KD}-Ras^{V12} (grey bars) acini exhibiting invasive protrusions following treatment with compounds indicated. Abbreviations: PD (MEK inhibitor PD98059), SP (JNK inhibitor SP600125), SB (p38α/β inhibitor SB202190). Black bars: pRS-Ras^{V12}, grey bars: Scribble^{KD}-Ras^{V12}. Error bars represent the s.e.m., calculated from three independent drug treatments. Statistics calculated using Student's *t*-test, $n=3$. (**c**) Western blot of whole-cell lysates from MCF10A cells lines treated as indicated for 24 h. (**d**) Images of pRS-Ras^{V12} and Scribble^{KD}-Ras^{V12} cells cultured in GFR Matrigel for 8 days in the presence of vehicle (DMSO) or PD98059 (2 μM). (**e**) Quantitation of invasion at day 8 in the presence of DMSO or 2 μM PD98059. Error bars represent s.e.m. Student's *t*-test, $n=5$ (no drug) and 2 (2 μM PD98059).

2005). Treatment with p38α/β or JNK inhibitors reduced cell invasion by twofold; however, the MEK inhibitor almost completely blocked invasion of Scribble^{KD}-Ras^{V12} cells (Figures 2a and b). Treatment of 3D cultures with 20 μM PD98059 not only inhibited cell invasion but markedly reduced cell proliferation, as indicated by acini growth. To avoid possible secondary effects of altering cell invasion by inhibiting proliferation we lowered the dosage of PD98059 (10-fold) to a level that reduced ERK phosphorylation below that of untreated cells, but did not completely inhibit it (Figure 2c). Treatment of 3D cultures with this dosage allowed normal growth of MCF10A acini but significantly inhibited cell invasion (Figures 2d and e), confirming that MEK-ERK signalling is essential for the invasion of Scribble^{KD}-Ras^{V12} cells.

Scribble regulates the activation of ERK

The dependence on MEK-ERK signalling and loss of Scribble for invasion of Ras^{V12} expressing cells raised the possibility that Scribble may directly regulate signalling through this pathway. Therefore, we measured the phosphorylation (activation) of the MAPK effectors ERK, JNK and p38 in Scribble-depleted cells. In asynchronously proliferating cells, depletion of Scribble caused a twofold increase in ERK activation, but did not affect phosphorylation of JNK1/2 or p38 (Figure 3a). Depletion of Scribble also resulted in an acute hyperphosphorylation of ERK in response to external growth factor stimuli (EGF, Figures 3b and c, Supplementary Figure 3) or transforming growth factor-β (TGFβ; Supplementary Figure 4). Interestingly, and in contrast to the 'steady-state' situation, Scribble depletion resulted in an increase in pJNK1 following EGF stimulation, consistent with the hyperactivation of JNK seen in *scribble* mutant *Drosophila* eye epithelium (Brumby and Richardson, 2003). Depletion of Scribble did not affect activation of p38 kinase (Figure 3b). Hyperphosphorylation of ERK and JNK following loss of Scribble was not specific to MCF10A cells as we observed a similar effect in 293T cells using an shRNA vector and small-interfering RNA (siRNA) oligos to different target sequences (Supplementary Figure 3). It should be noted that the activity of the MAPK pathway in Scribble^{KD} cells is dependent on the presence of growth factor stimuli and these cells do not show growth factor independent proliferation, induction of p21 or morphological effects characteristic of expression of active MAPK pathway oncogenes such as Raf (Woods *et al*., 1997; Lehmann *et al*., 2000). Importantly, in cells expressing Ras^{V12}, Scribble loss caused increased activation of ERK (Figure 3a), indicating that despite the V12 mutation, Ras alone does not activate MAPK signalling at maximal levels in these cells. We noted, as previously published, that increasing ERK activation in Ras^{V12} cells (using TGFβ) can induce cell invasion (Supplementary Figure 4; Seton-Rogers and Brugge, 2004; Kim *et al*., 2005). Scribble depletion in this context induced a striking hyperactivation of ERK (but not JNK or p38) and further increased cell invasion in 3D cultures (Supplementary Figure 4), underscoring the importance of the absolute level of MAPK activation in determining cellular response.

Scribble suppresses Ras-mediated cell invasion and disruption to cell polarity

The above data indicated that Scribble directly modulates MAPK signalling, so we asked whether Scribble could act to antagonize oncogenic Ras (V12) signalling, thus preventing cell transformation. As shown earlier, moderate expression of Ras^{V12} in MCF10A cells led to a distinct 'fibroblastic' change in cell shape (Figure 4a, upper left). Overexpression of Scribble (Figure 4b) completely blocked this change and cells retained a cobblestone morphology (Figure 4a, upper right). Given this, we asked whether Scribble could affect the behaviour of Ras^{V12} cells in 3D culture. Expression of Ras^{V12} was not sufficient to induce invasion in Matrigel culture (Figure 1); however, we noted that the addition of collagen I (1.6 mg/ml) to the 3D cultures stimulates invasion of these cells. Consistent with the observation that loss of Scribble enhanced invasion of Ras-transformed MCF10A cells, increased expression of Scribble completely suppressed the development of invasive protrusions in cells expressing Ras^{V12} alone, from 15.7 ± 4.1 to $0.8 \pm 0.6\%$ (Figure 4c). Scribble expression not only inhibited cell invasion, but also partially prevented the loss of acinar organization (Figure 4d, left) and the loss of polarity (Figure 4d, right) caused by Ras^{V12}. The loss of epithelial morphology in MCF10A cells expressing Ras^{V12} correlated with a dramatic loss of E-cadherin mRNA and protein, which was restored by the expression of Scribble (Figures 4e and f). Scribble also restored the expression of *CRB3*, a polarity regulator that is reduced during the epithelial to mesenchymal transition (Aigner *et al*., 2007) in Ras^{V12} expressing MCF10A cells (Figure 4f).

Scribble suppresses Ras-dependent anchorage-independent growth and MAPK target gene expression

The strong suppression of the Ras^{V12} phenotype suggested that Scribble might not only control cell invasion, but also may act more generally to control many or all of the events downstream of Ras signalling. To test this, we analysed the effect of increased Scribble expression on: (1) the phosphorylation (activation) of downstream effector pathways and (2) the expression of Ras-MAPK target genes. MCF10A expressing Ras^{V12} were responsive to EGF stimulation and downstream kinases ERK, JNK and Akt were rapidly phosphorylated following addition of EGF to starved (Figure 5a) or unstarved (not shown) cultures. We did not observe an increase in p38 kinase phosphorylation (not shown). Scribble significantly inhibited the activation of ERK1/2 and JNK1, but did not alter the activation of Akt1 (Figure 5a), suggesting a specific role for Scribble in mediating a subset of Ras-mediated signalling events. We next asked whether Scribble expression would suppress the transcriptional changes induced by Ras^{V12}. Earlier we showed that Scribble could block the Ras^{V12}-

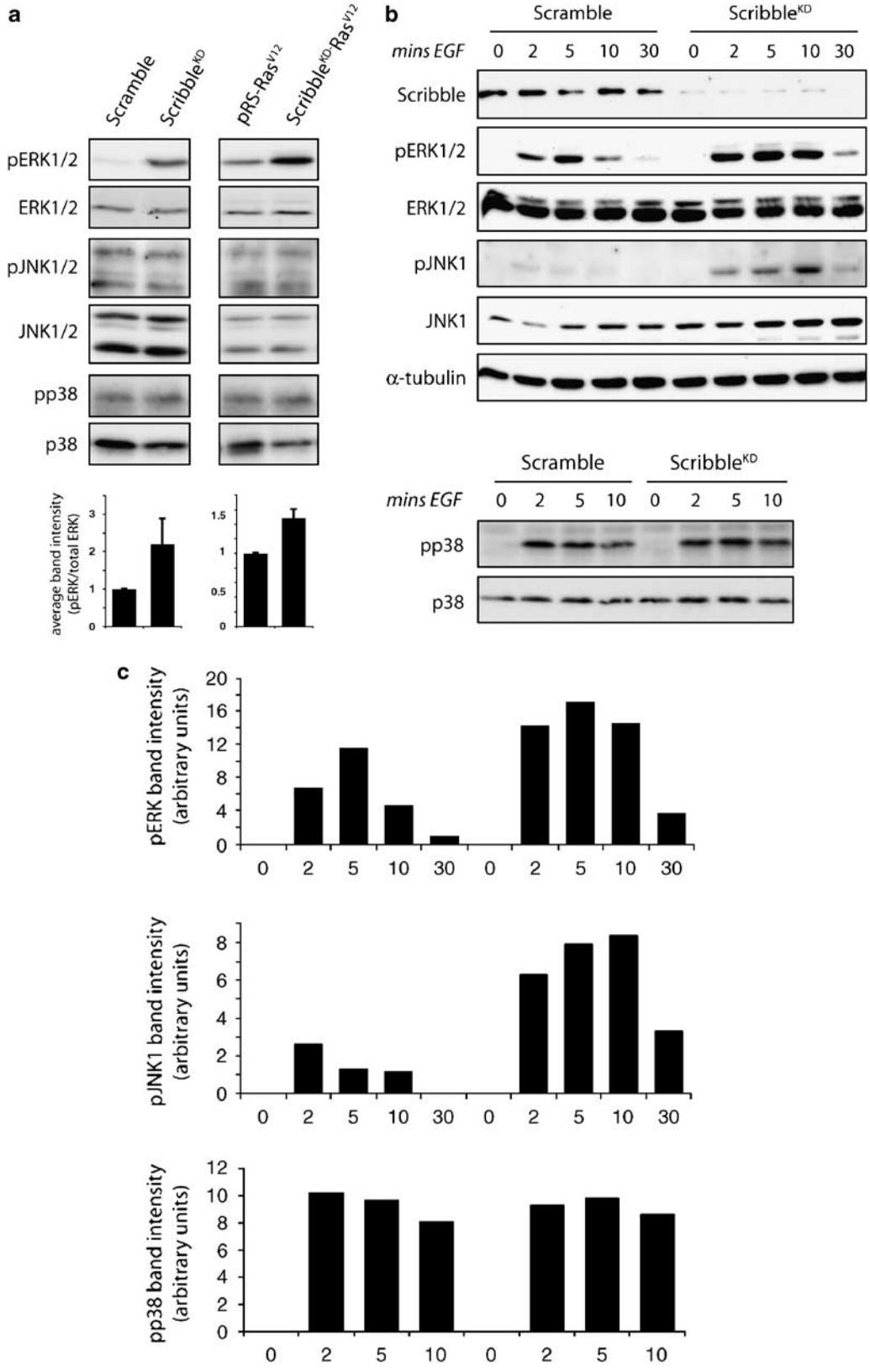


Figure 3 Scribble regulates phosphorylation of extracellular-regulated kinase (ERK) and Jun N-terminal Kinase (JNK), but not p38. (**a**) Western blots of whole-cell lysates from asynchronously proliferating, sub-confluent stable MCF10A cell lines, as indicated. Shown beneath is the mean level of pERK in each cell population, plotted relative to control cells. Error bars represent s.e.m., $n = 5$. (**b**) Western blots of whole-cell lysates from stable MCF10A cell lines, as indicated, stimulated with 20 ng/ml epidermal growth factor (EGF) for 0, 2, 5, 10 and 30 min and blotted with antibodies as indicated to the left of each panel. The pp38/p38 blot shown beneath represents the same cell lysates as shown above. (**c**) Graphs represent densitometry of western blots in (**b**), showing phospho-protein abundance at each time point following EGF stimulation.

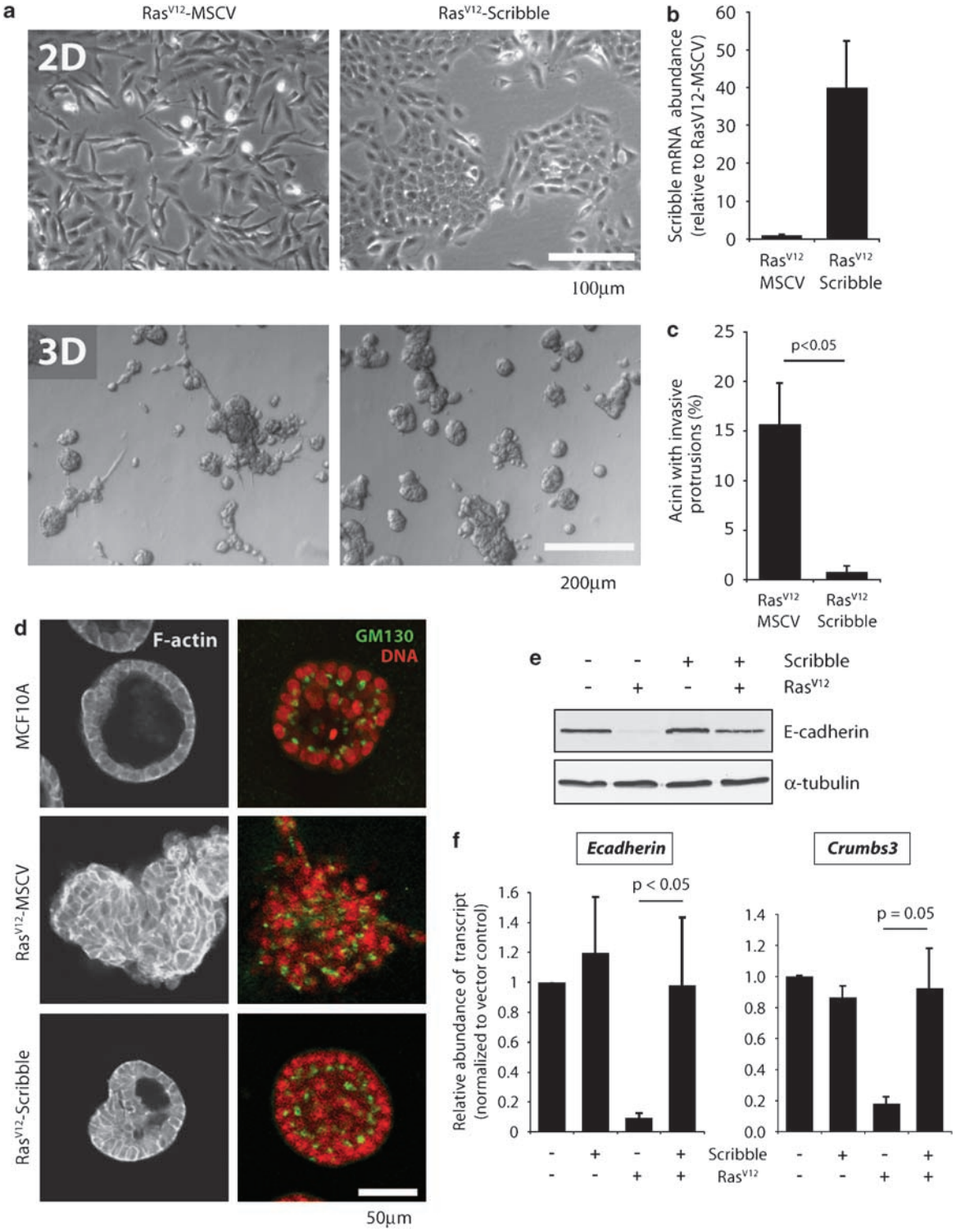


Figure 4 Scribble overexpression suppresses Ras-mediated cell invasion and disruption to acinar polarity. (**a**) Images of Ras^{V12}-MSCV and Ras^{V12}-Scribble MCF10A cells cultured in 2D or 3D in Matrigel/collagen I for 8 days. (**b**) Abundance of *scribble* mRNA in Ras^{V12}-MSCV and Ras^{V12}-Scribble cells as determined by quantitative RT-PCR. (**c**) Quantitation of invasion in 3D cultures calculated at day 8 of culture. Graph represents the means of three independent experiments ± s.e.m. ($n=3$, $P<0.05$, Student's *t*-test). (**d**) Confocal images of control, Ras^{V12}-MSCV and Ras^{V12}-Scribble at day 16 of 3D culture. (Left) Acini are labelled with phalloidin to stain F-actin and highlight acinar structure. (Right) Acini are labelled with propidium iodide (DNA, red) and immunostained with α-GM130 (green) to mark the Golgi apparatus. (**e**) Western blot of whole-cell lysates from stable MCF10A cell lines. (**f**) Relative abundance of *E-cadherin* and *Crb3* mRNA determined by quantitative RT-PCR and normalized to the vector control cell population. Error bars represent s.e.m., three independent experiments performed in duplicate (Student's *t*-test, $n=3$).

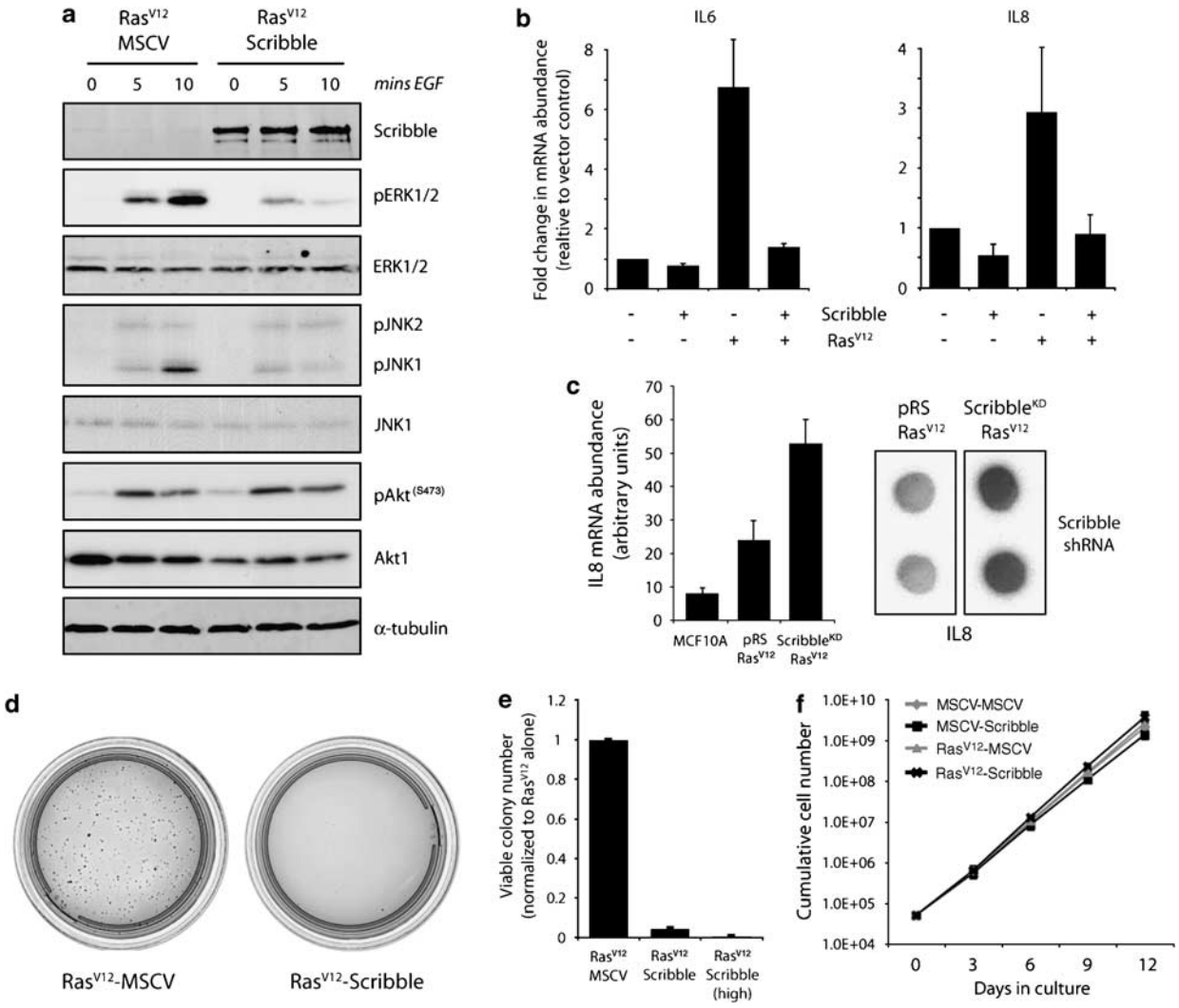


Figure 5 Scribble suppresses mitogen-activated protein kinase (MAPK) activation, Ras target-gene expression and anchorage-independent growth in soft agar. (**a**) Western blot of whole-cell lysates from MCF10A stable cell lines, epidermal growth factor (EGF) starved for 24 h and stimulated with 20 ng/ml EGF for 0, 5 and 10 min as indicated. (**b**) Relative abundance of interleukin (IL)-6 and IL-8 mRNA transcripts determined by quantitative RT-PCR and plotted relative to the mRNA abundance in vector control MCF10A cells. Graph shows mean values of two independent experiments performed in duplicate ± s.e.m. (**c**, left) Relative abundance of IL-8 mRNA transcript determined as above in pRS-Ras^{V12} and Scribble^{KD}-Ras^{V12} (Scribble knockdown-Ras^{V12}) cell lines, plotted relative to control MCF10A cells. Graph shows mean values of two independent experiments performed in triplicate ± s.e.m. (Right) Detection of secreted IL-8 in the supernatant of cultured MCF10A cell lines indicated. Blots correspond to duplicate IL-8 detection spots on a cytokine array, as described in Supplementary Text. (**d**) Photographs of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-stained soft agar cultures at day 12. (**e**) Graph shows the number of viable colonies 12 days after plating, normalized to Ras^{V12}-MSCV alone. (**f**) Graph of cumulative cell number of each of the cell populations indicated. Error bars are the standard deviation of the mean ($n = 3$).

mediated repression of E-cadherin (Figure 4). Ras activation also promotes the expression of many cytokines and growth factors necessary to maintain the transformed phenotype. Two such factors are interleukin (IL)-6 and IL-8, pro-inflammatory cytokines that are crucial for Ras-driven tumourigenicity in xenograft models (Park *et al*., 2003; Sparmann and Bar-Sagi, 2004). IL-6 and IL-8 were induced sixfold and threefold, respectively, following expression of Ras^{V12}, and Scribble expression suppressed the induction of both mRNA transcripts to near wild-type levels (Figure 5b). This effect was dependent on the level of Scribble protein because cells expressing higher levels of Scribble showed greater suppression of IL8 mRNA (Supplementary Figure 5). Moreover, in the presence of activated Ras, loss of Scribble enhanced the abundance of IL-8 transcript (Figure 5c, left) and this translated to an elevated secretion of IL-8 protein, measured using an enzyme-linked immunosorbent assay-based detection assay (Figure 5c, right). Thus, Scribble has a dose-dependent, inhibitory effect on Ras target-gene expression.

A classic *in vitro* measure of cell transformation is the ability of cells to form colonies in semi-solid media. In immortalized MCF10A cells, Ras^{V12} is sufficient to promote growth in soft agar (Figure 5d, left). In

contrast, cells also expressing Scribble showed a significant 20-fold decrease in the efficiency of colony formation in soft agar (Figure 5d, right). This effect was dose-dependent as cells expressing higher levels of Scribble showed a further reduction in colony formation (Figure 5e). This decrease in colony forming ability was not a result of decreased cell proliferation in the presence of Scribble, as we observed that while expression of Scribble in non-transformed cells reduces cell proliferation, in the presence of Ras^{V12}, Scribble caused no reduction in cell proliferation in 2D culture (Figure 5f). Together, these data define an important role for Scribble in regulating the cellular response to Ras-MAPK signalling in mammalian epithelial cells.

Loss of Scribble promotes cell invasion in cooperation with Raf-MEK-ERK signalling

We noted earlier that activation of MEK-ERK through Ras^{V12} was not sufficient to induce cell invasion in Matrigel. However, as Scribble restricts MAPK signalling in the presence of Ras^{V12}, we hypothesized that loss of Scribble might cooperate with events that promote activation of the Raf-MEK-ERK pathway. To directly test this we specifically activated Raf-MEK-ERK signalling in Scribble-depleted cells using a previously described GFP-RafΔ22W-ER (RafER) fusion protein that is activated by the addition of 4-hydroxy-tamoxifen (4OHT) to the culture media (Woods *et al*., 1997). Activation of RafER was not sufficient to induce complete loss of E-cadherin and epithelial cells junctions such as Ras^{V12}; however, $Scribble^{KD}$-RafER cells appeared more fibroblastic as compared with those expressing RafER alone and showed reduced E-cadherin protein levels (Figures 6a and b). When cultured in a Matrigel/collagen I matrix in the presence of 4OHT, RafER cells showed a low level of invasion, similar to that seen with Ras^{V12} in the same 3D matrix (Figures 6c and d). $Scribble^{KD}$-RafER cells showed a threefold increase in invasion (20.5 ± 7.8 vs $58.7 \pm 17.3\%$). $Scribble^{KD}$-RafER cells also showed increased invasion when 4OHT-naive cells were treated with 4OHT at the beginning of 3D culture, albeit to a lower level (data not shown), suggesting chronic activation of Raf provides additional factors to promote invasion, as previously reported (Lehmann *et al*., 2000). Conversely, increased expression of Scribble suppressed the Raf-dependent reduction in E-cadherin expression (Figure 6e) and reduced expression of IL-8 following the activation of Raf (Figure 6f), similar to that observed in cells expressing Ras^{V12}.

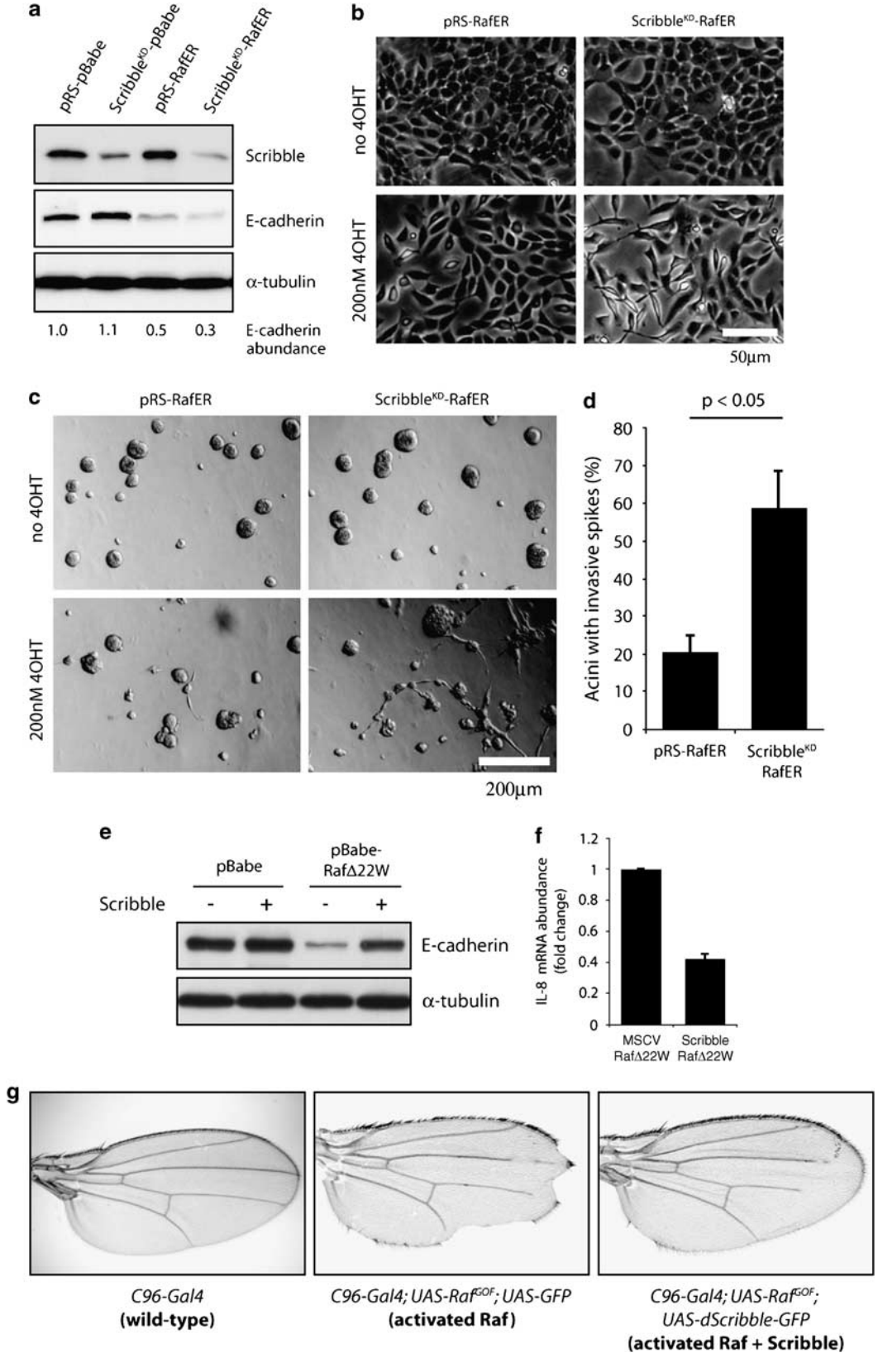
We next wished to determine whether suppression of MAPK signalling was a conserved function of Scribble. For this we turned to *Drosophila*, where cooperation between Ras-Raf-MAPK signalling and loss of Scribble was first described (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). To examine the effects of Scribble on Raf-MAPK signalling, we expressed a *raf* gain-of-function (raf^{GOF}) allele in the *Drosophila* wing margin using a *C96-GAL4* driver. Using this driver, expression of activated alleles of *dEGFR* and (rat) *NeuNT*, which promote Ras-MAPK signalling, results in notching in the posterior compartment of the adult wing (Settle *et al*., 2003). Expression of Raf^{GOF} in the wing margin produced a similar phenotype (Figure 6g, middle). Importantly, expression of *Drosophila* Scribble in the presence of Raf^{GOF} almost completely restored the normal structure of the adult wing (Figure 6g, right). Similarly, *Drosophila* Scribble could also suppress the wing defects resulting from Ras^{GOF} expression (data not shown). Thus, Scribble can repress the effects of constitutive MAPK activation in *Drosophila*, indicating a conserved role for Scribble in regulating MAPK signalling.

Discussion

Here we describe a functional interaction between the mammalian polarity regulator Scribble and the MAPK signalling pathway. We show that Scribble prevents invasion of Ras^{V12}-transformed human mammary epithelial cells by blocking the downstream effects of MAPK signalling. This data extend the hypothesis drawn from early *Drosophila* studies, which showed that loss of *scribble* promotes spontaneous cell invasion and metastasis of epithelial cells (Brumby and Richardson, 2003; Pagliarini and Xu, 2003). The *Drosophila* model proposes that Scribble restricts invasion through negative regulation of a pathway separate to Ras signalling, in that context, the JNK pathway. However, our data show that while Scribble can regulate JNK activity following acute EGF (but not TGFβ) stimulation, it primarily controls cell transformation and invasion by direct suppression of Raf-MEK-ERK signalling events downstream of Ras^{V12}. It is not clear why JNK activation is only affected by Scribble following acute EGF signals, but it is unlikely to be a

Figure 6 Loss of Scribble promotes invasion of cells expressing-activated Raf. (**a**) Western blot of lysates from cells cultured in the presence of 200 nM 4-hydroxy-tamoxifen (4OHT). Numbers below the blot indicate the relative abundance of E-cadherin protein relative to pRS-MSCV cells, quantitated by densitometry from three independent protein samples. (**b**) Phase contrast images of MCF10A cells listed cultured for 14 days with or without 200 nM 4OHT. (**c**) Phase contrast images of MCF10A cells cultured in Matrigel/collagen I for 8 days with or without 200 nM 4OHT. (**d**) Quantitation of cell invasion in 3D culture. Graph represents the mean of three independent experiments ± s.e.m. ($n = 3$, $P < 0.05$, Student's *t*-test). (**e**) Western blot of whole-cell lysates from cells indicated. (**f**) Relative abundance of IL-8 mRNA transcript in cell populations indicated, expressed as a fold change, normalized to MSCV-RafΔ22W. Data are the average of three independent experiments. (**g**) Representative images of *Drosophila* adult wings from *C96-GAL4* control (left), *C96-GAL4*; *UAS-*Raf^{GOF}; *UAS-GFP* (middle) and *C96-GAL4*; *UAS-*Raf^{GOF}; *UAS-dScribble-GFP* (right) animals. A simplified description of each genotype is shown beneath.

result of cross talk between ERK and JNK downstream of MEK as inhibition of MEK1/2 does not block EGF-induced activation of the JNK pathway (data not shown). We are currently investigating whether JNK regulation is in some way functionally compromised in Scribble-depleted cells.



GTP-bound Ras activates many effector pathways, including PI3K-Akt, RalGDS-Ral and the MAP kinase effectors ERK, JNK and p38. In particular, ERK and p38 activation have been reported to be responsible for invasion in Ras-transformed MCF10A cells (Kim *et al*., 2003; Seton-Rogers *et al*., 2004). Here, ERK activation but not p38 is required for the invasion of Scribble^{KD}-Ras^{V12} MCF10A cells. This is consistent with recent studies showing factors that activate MAPK signalling (Ras, ErbB2 and TGFβ) cooperate to induce invasion of MCF10A cells (Giunciuglio *et al*., 1995; Seton-Rogers *et al*., 2004). *In vivo*, high level activation of Ras-MAPK in normal mouse mammary epithelium induces cell senescence (Sarkisian *et al*., 2007); however, in cells lacking key senescence triggers (for example, p53-Ink4a-Arf), increased Ras signalling promotes invasive and aggressive disease (Aguilar-Cordova *et al*., 1991; Sarkisian *et al*., 2007). Thus, events that modulate the level of MAPK signalling can significantly affect the progression of cancers harbouring activating Ras mutations.

We and others have previously shown that Scribble positively regulates migration in mammalian cells (Osmani *et al*., 2006; Dow *et al*., 2007); however, here we show that Scribble acts to restrict cell invasion, suggesting the role of Scribble in controlling cell movement is context dependent. Consistent with a context-dependent or cell-type-specific role, in canine epithelial (MDCK) cells, Scribble appears to negatively regulate sheet migration (Qin *et al*., 2005). It is not clear what controls the switch between these pro-migratory and anti-invasive functions, but many polarity proteins seem to play a central role in both processes, perhaps through a common mechanism. Polarity regulators of the Scribble-Dlg-Lgl and Par3-Par6-aPKC groups promote directed cell migration by spatially coordinating and restricting extracellular signals and intracellular GTPase signalling components to the leading edge (Humbert *et al*., 2006). Similarly, the role for Scribble and other polarity proteins in restricting cell transformation and invasion could lie in the regulation of receptor-mediated signalling. In addition to the regulation of MAPK signalling shown here, in other systems Scribble is known to coordinate trafficking of transmembrane receptors (Lahuna *et al*., 2005) and the Par6-aPKC complex has been shown to control both cell protrusion and cell multi-layering through interaction with TGFβR and ErbB2 in epithelial cells (Ozdamar *et al*., 2005; Aranda *et al*., 2006). Interestingly, the effect of Par6 on ErbB2-dependent cell multi-layering is Scribble dependent (Aranda *et al*., 2006), suggesting the complexes may functionally interact to control cell transformation, as they do during directed migration. Although the precise downstream effectors mediating this have not yet been identified, MEK-ERK is an attractive candidate. Two recent papers have shown that another polarity regulator, Par3, both promotes directed migration in keratinocytes (Pegtel *et al*., 2007) and prevents the formation of Rac-dependent lamellipodia and filopodia in neurons (Zhang and Macara, 2006) by appropriately directing the subcellular localization of the RacGEF, Tiam1. Similarly, Scribble controls RacGEF βPix and Rac1 localization during directed migration (Dow *et al*., 2007); however, our experiments suggest that deregulation of Rac1 activity is not responsible for the increased invasion of Scribble-depleted cells (Supplementary Figure 6), although we cannot exclude a change in the activation of other GTPases or polarity proteins that regulate downstream MAPK signalling. It should be noted that the environmental context significantly affects invasive cell behaviour, as we did not observe any increase in cell motility in Scribble^{KD}-Ras^{V12} cells in 2D scratch assays (data not shown).

Our data suggest that Scribble acts downstream of Ras and Raf (as it can block their phenotypic effects), but upstream of ERK as inhibition of MEK can suppress the effects of Scribble loss (at least in the context of Ras^{V12}). Notably, Scribble exists in a complex with the Arf-GAP GIT1, a known positive modulator of MEK-ERK signalling (Audebert *et al*., 2004; Yin *et al*., 2004) and thus could act as a negative regulator of GIT1 activity. Although strongly associated with GIT1 (not shown), we have not observed any physical interaction between Scribble and members of the MAPK pathway. However, such interactions may be indirect or occur only transiently. Qin *et al*. (2005) recently showed that loss of Scribble in MDCK cells could disrupt AJs, which are known to scaffold many signalling networks, including components of the MAPK pathway (McLachlan and Yap, 2007). While the role of Scribble in regulating AJs may affect invasive cell behaviour in some contexts, our data suggest that Scribble controls MAPK activity independently of AJs as ERK is hyperphosphorylated in both Scribble-depleted MCF10A cells that maintain junctions (Dow *et al*., 2007) and 293T cells, which do not form stable AJs.

Numerous recent reports have described that loss of Scribble and Dlg is correlated with late stage, aggressive epithelial tumours, including breast tumours (Massimi *et al*., 2004; Nakagawa *et al*., 2004; Navarro *et al*., 2005; Gardiol *et al*., 2006); however, there remains a seeming contradiction about the role of the Scribble-Dlg-Lgl complex in cancer progression. Dlg is known to be required for transformation by Ad9 E4-ORF1 (Frese *et al*., 2006) and Dlg is reported to have increased expression in low-grade tumours, but significantly decreased levels in high-grade cervical neoplasias (Lin *et al*., 2004). In addition, both Dlg and Scribble show increased expression in well-differentiated colon adenocarcinomas (Gardiol *et al*., 2006; Kamei *et al*., 2007). Together these data suggest that Scribble might play a dual role in tumourigenesis, perhaps being required at early stages of tumour development but blocking the progression to invasive disease. Here we have shown that Scribble can suppress Ras-driven cell invasion as well as the production of pro-tumourigenic cytokines such as IL-8. However, we also noted that Scribble overexpression causes a mild increase in cell proliferation in the presence of activated Ras (Figure 5f). Thus, in some cases high Scribble expression may be pro-proliferative and thus, pro-tumourigenic, whereas it

also acts to restrict tumour progression by inhibition of cell invasion and by blocking the production of pro-angiogenic cytokines (that is, IL-8). The use of conditional knockout *scribble* alleles and regulatable RNAi technology will provide the best means to provide a definitive understanding of the role of Scribble in both tumour establishment and evolution *in vivo*.

The initial identification of cooperation between oncogenic Ras and loss of Scribble in *Drosophila* offered the enticing suggestion that polarity regulators could have functional importance in the development of human cancers. Here we have shown that Scribble functions in human cells to restrict cell transformation and invasion and have defined Scribble as a new node that regulates Ras-Raf-MAPK signalling.

Materials and methods

Reagents
4-Hydroxy-tamoxifen (Sigma-Aldrich, St Louis, MO) was used at 200 nM (diluted from 2 mM stock in ethanol) to activate RafER fusion protein. Conditions for studies with chemical inhibitors: MEK inhibitor, PD98059 (20 or 2 μM; Sigma-Aldrich); JNK inhibitor, SP600125 (20 μM; Calbiochem, San Jose, CA, USA); p38 inhibitor, SB202190 (20 μM; Calbiochem). All compounds used were solubilized in dimethyl-sulphoxide. The following primary antibodies were used: α-Scribble (7C6-D10), mouse monoclonal (Dow *et al*., 2003); α-E-cadherin (no. C20820), α-GM130 (no. 6108222) and α-JNK1/2 (no. 554285; BD Biosciences, San Jose, CA, USA); α-Ha-Ras (no. 05-775; Upstate Biotechnologies—Millipore, Billerica, MA, USA); α-tubulin (no. T5168; Sigma-Aldrich); α-pERK1/2 (no. 9106), α-ERK1/2 (no. 9102), α-pJNK1/2 (no. 9251), α-pp38 (no. 9211), α-p38 (no. 9212) and α-pAkt1 (no. 9271; Cell Signalling Technologies, Beverly, MA, USA); α-panAkt (gift from Dr R Pearson, Peter MacCallum Cancer Centre; affinity purified sheep polyclonal raised against the C-terminal 16 residues of Akt1). Fluorescent dyes: Alexa-647-phalloidin and ToPRO-3 iodide (no. T3605; Molecular Probes, Eugene, OR, USA). α-Mouse IRDye 800 (no. 610-732-124; Rockland Immunochemicals Inc., Gilbertsville, PA, USA) was used for LiCor western blotting. All other fluorescent secondary antibodies for indirect immunofluorescence and LiCor western blotting were purchased from Molecular Probes. All details of *Drosophila* experiments are available as Supplementary Information.

Cloning and expression constructs
Small-hairpin RNA retroviral constructs were constructed in pRETROSuper, using target sequences: shScribble: gtcattggaacaggacgct; shScramble: agtactgcttacgatacgg. Human Scribble siRNA oligos (catalogue no. 16708) and negative controls (catalogue no. 4611) were purchased from Ambion Inc. (Austin, TX, USA). H-Ras^{V12} was cloned into MSCV-IRES-GFP and MSCV-IRES-Cherry using an *Eco*RI restriction fragment from pBabe-H-Ras^{V12} (Dr David Tuveson, Cambridge Research Institute, Cancer research, UK). A modified KIAA0147 clone containing the full-length Scribble cDNA (previously described in (Dow *et al*., 2003)) was cloned (*Bam*HI/*Not*I, blunt) into MSCV-IRES-GFP (*Eco*RI, blunt) and pBabe-Hygromycin (*Bam*HI, blunt). Murine Scribble was cloned from mKIAA0147 (Kasuza DNA Institute) using a *Kpn*I-*Dra*I (blunt) restriction fragment into MSCV-IRES-GFP (*Eco*RI, blunt). pBabe-GFP-RafΔ22W-ER (Dr Michael Olson, Beatson Institute of Cancer Research, Glasgow) and pBabe-RafΔ22W (Dr Christopher Counter, Duke Unversity Medical Center, NC, USA) have been previously described (Stanton *et al*., 1989; Woods *et al*., 1997).

Cell culture
MCF10A cells were cultured in 2D as previously described (Debnath *et al*., 2003; Dow *et al*., 2007). Three-dimensional MCF10A organotypic cultures were performed using the 'overlay method' as previously described (Debnath *et al*., 2003). For collagen I/Matrigel 3D cultures, rat-tail collagen type I (BD Biosciences) was pH neutralized using 5 × Dulbecco's modified Eagle's medium to a concentration of 3.2 mg/ml. Growth factor-reduced (GFR) Matrigel was combined 1:1 with neutralized collagen and mixed thoroughly on ice. The final collagen I concentration was 1.6 mg/ml. Media was replaced every 4 days for the duration of the culture period. For inhibitor studies in 3D, the drug was replaced every 2 days. For immunofluorescent staining, cultures were processed essentially as described previously (Debnath *et al*., 2003). Details for EGF stimulation experiments, quantitative RT-PCR and soft agar culture are available as Supplementary Text.

Image acquisition and manipulation
Details of image acquisition and post-processing are available as Supplementary Text.

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Contributions: LED designed, performed and analysed experiments and wrote the paper. IAE performed and analysed experiments. CLK performed and analysed experiments. KMK performed experiments. HER designed and performed and analysed experiments. POH designed experiments and wrote the paper.

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