Localization, Not Important in All Tumor-Suppressing Properties: A Lesson Learnt from Scribble

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Key Words

Scribble · Protein localization · Mitogen-activated protein kinase · Epithelial to mesenchymal transition · Transformation · Epithelia · MCF10A

Abstract

Aberrant localization of proteins is increasingly being suggested as a causal player in epithelial cancers. Despite this, few studies have investigated how mislocalization of a protein can alter individual biological processes that contribute to cancer progression. Using Ras as a model of transformation, we investigate how localization of the polarity protein

Scribble contributes to its tumor-suppressive properties. Wild-type Scribble has been shown to modulate Ras-mitogen-activated protein kinase (MAPK) transformation both in vitro and in vivo. By utilizing a construct that carries a mutation in the LRR domain of Scribble (Scribble P305L) resulting in a cytosolic rather than the usual membrane-bound localization, we report that discrete tumor suppressive properties of Scribble are differentially sensitive to the localization of Scribble. We find that although the Scribble P305L mislocalization mutant can no longer suppress Ras-MAPK-induced invasion or epithelial to mesenchymal transition phenotypes, mislocalized Scribble can still suppress anchorage-independent cell growth. This study illustrates that the man-

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Abbreviations used in this paper

EMT	epithelial to mesenchymal transition
ERK	extracellular signal regulator
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
Scrib P305L	Scribble P305L
Scrib wt	Scribble wild type

ner in which protein mislocalization contributes to cancer is likely complex and highlights the need for careful interrogation as to how cell polarity protein mislocalization, its secondary consequences, and the mutations that give rise to their mislocalization may contribute to specific aspects of cancer progression.

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Introduction

Correct subcellular localization of proteins is critical for their function and for accurate activation of the appropriate pathways by providing physiological context. Many factors can lead to abnormal protein localization, including mutations and/or defects in protein trafficking machinery that often result in either activation or deactivation of signaling pathways. Aberrant or modified localization can result in a variety of disorders and diseases, including metabolic, cardiovascular, neurodegenerative and cancer [Hung and Link, 2011].

Numerous cell polarity proteins, including PKC λ / and members of the Scribble complex, Scribble, Discs Large and Lethal Giant Larvae have all been identified as being mislocalized in various cancer types [Nakagawa et al., 2004; Eder et al., 2005; Gardiol et al., 2006; Zhan et al., 2008; Lisovsky et al., 2009; Huang and Muthuswamy, 2010; Pearson et al., 2011; Martin-Belmonte and Perez-Moreno, 2012]. Scribble in particular has been identified as mislocalized in several cancer types, including breast, prostate, cervical and colon cancer [Nakagawa et al., 2004; Gardiol et al., 2006; Zhan et al., 2008; Pearson et al., 2011]. In normal tissue Scribble is located at the membrane whilst in cancer tissue it is sometimes found in cytosolic pools. Scribble was originally identified in *Drosophila melanogaster* as a neoplastic tumor suppressor with loss of function disrupting apico-basal polarity, junctional integrity, and inducing inappropriate proliferation and tissue overgrowth [Bilder and Perrimon, 2000]. Since then, human Scribble has been shown to act as a functional homologue of *Drosophila* Scribble [Dow et al., 2003] and has been implicated in numerous cellular processes including proliferation, differentiation, apoptosis, stem-cell maintenance, migration and vesicle trafficking [Humbert et al., 2008].

Scribble belongs to the LAP family, which describes proteins that contain either 1 or 4 PSD95/Discs Large/ZO-1 (PDZ) domains and a leucine-rich repeat (LRR) region, of which Scribble has 16, and functions in control-

ling cell shape, size and subcellular protein localization [Bryant and Huwe, 2000]. Scribble contains 4 PDZ domains, which are regions of 80–90 amino acids, found ubiquitously across the animal kingdom that act through protein-protein interactions whilst the LRR domain tether the protein to the plasma membrane [Santoni et al., 2002; Legouis et al., 2003]. The different domains of Scribble are mutually dependent on each other for efficient activity. Studies in *Drosophila* Scribble have found the LRR domain necessary for both cell polarity and control of cell proliferation; however, cooperative activity between the LRR and the PDZ domains is required to achieve full function [Zeitler et al., 2004].

We and others have shown that in addition to Scribble expression levels being up- or downregulated, Scribble can be mislocalized in human cancer and this has been proposed to be a causal event [Nakagawa et al., 2004; Gardiol et al., 2006; Zhan et al., 2008; Pearson et al., 2011]. In particular, in prostate cancer where Scribble levels are elevated [Pearson et al., 2011] or in breast cancer where Scribble has been observed to be overexpressed [Vaira et al., 2011], overexpression of a mislocalized version has been proposed to contribute to its tumor-promoting properties. To begin to address how mislocalized Scribble contributes to tumorigenesis, we have used a Scribble mislocalization construct to study its effects on transformation and invasion in the nontransformed human breast cell line MCF10As. The mislocalization construct used carries a point mutation of proline 305 to leucine in the thirteenth LRR region causing mislocalization away from the plasma membrane to the cytoplasm [Legouis et al., 2003; Audebert et al., 2004]. A similar mutation has been studied in LET-413 (the homologue of Scribble in *Caenorhabditis elegans*) where it is also required for correct basolateral localization and for LET-413 function during embryogenesis [Legouis et al., 2003]. Mutations in the LRR domain of Scribble have also been observed in mice such as in the *line-90* mouse where mutation I285K results in perinatal death due to morphological defects including craniorachischisis and exencephaly [Zarbalis et al., 2004]. Fetal liver cells and embryonic keratinocytes from *line-90* mice show marked mislocalization of Scribble protein away from the membrane [our unpubl. data; Dow et al., 2007]. Additionally, in human patients presenting with craniorachischisis, mutations resulting in aberrant protein localization of Scribble have also been documented [Robinson et al., 2012].

In this study we focus on the functional biological outcomes that result from a Scribble mislocalization mutant.

We show that unlike wild-type human Scribble that can suppress the mitogen-activated protein kinase extracellular signal regulator (MAPK-ERK) signaling cascade, the P305L mislocalization mutant is unable to do so. As the Ras-MAPK signaling pathway is often deregulated in cancer, we use a Ras^{V12} model of transformation to examine the effect of Scribble expression and its localization in this context. We find that wild-type Scribble exerts several tumor suppressive effects, including restoration of E-cadherin and suppression of invasion and anchorage-independent cell growth. In contrast, although the Scribble P305L (Scrib P305L) mislocalization mutant could restore colony growth in soft agar similarly to wild-type Scribble and could partially restore E-cadherin expression, the Scribble mislocalization mutant resulted in sustained upregulation of MAPK-ERK signaling and failed to suppress invasion, thus acting similarly to a loss of function allele. Thus, Scribble localization accounts for only some of its tumor suppressive properties. These studies highlight the need for careful interrogation as to how protein mislocalization, in particular that of cell polarity proteins, contributes to specific aspects of cellular transformation and tumorigenesis.

Material and Methods

Cell Maintenance

MCF10A cells were maintained in DMEM:F12 (Dulbecco's Modified Eagle Medium: F12) supplemented with 5% donor horse serum (Gibco, Grand Island, N.Y., USA), 10 µg/ml insulin (Novo Pharmaceuticals, Crawley, UK), 0.5 µg/ml hydrocortisone (Sigma Aldrich, St. Louis, Mo., USA), 20 ng/ml EGF (Cytolab Ltd, Dällikon, Switzerland), 100 ng/ml cholera toxin (Sigma Aldrich), penicillin (100 U/ml) and streptomycin (100 U/ml) as previously described [Debnath et al., 2003]. 293T cells were maintained in DMEM supplemented with 10% fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 U/ml). All cultures were maintained at 37°C in 5% CO₂.

Expression Constructs

The pLPCX Scrib P305L was a kind gift from Prof. Senthil Muthuswamy (Ontario Cancer Institute, Canada) and has been described previously [Zhan et al., 2008]. A fragment of the modified KIAA00147 clone containing human Scribble cDNA with wild-type LRR domains, previously described elsewhere [Dow et al., 2003], was cloned with HindIII and SphI into pLPCX Scrib P305L to create the pLPCX Scribble wild-type (Scrib wt) vector. MSCV-IRES-cherry-H-Ras^{V12} was cloned from pBabe-H-Ras^{V12} as previously described [Dow et al., 2008]. All constructs were verified by sequencing.

Generation of Stable Cell Lines

Virus was generated by transfecting 293T cells by calcium phosphate precipitation. The day prior to transfection, 293T cells were plated at a density of 10⁴ cells/cm². Transfection was carried out by combining vector DNA (10 µg) with the amphotropic packaging vector RD114 (10 µg) in 1 ml CaCl₂ (0.25 M final) and mixing with an equal volume of Hepes-buffered saline (0.3 M NaCl, 0.06 M Hepes, 0.45 mM Na₂HPO₄, pH 7.10). The mixture was added dropwise to cell cultures. Approximately 18 h following transfection cells were washed with PBS and fresh MCF10A media applied. Viral supernatant was collected every 12 h, filtered using 0.22-µm filters and applied directly to MCF10A cells for generation of stable lines. For transduction, MCF10A cells were plated at 5 × 10⁴ cells/well in a 6-well plate 24 h prior to the initial viral hit. Virus was applied 3 times at 12-hour intervals and allowed to recover for 24 h before selection. To generate a polyclonal stable cell population, cells were either selected for 1 week in 2 µg/ml puromycin and/or sorted for cherry⁺ cells on a FACStar flow cytometer (Becton Dickinson, Franklin Lakes, N.J., USA).

3D Organotypic Culturing

Organotypic 3D MCF10A cultures were performed using the overlay method as previously described [Debnath et al., 2003] with the exception that cells were seeded onto a Matrigel:Collagen bed. Briefly, Bovine Collagen (PureCol™, Advanced BioMatrix, San Diego, Calif., USA) was neutralized with 10 mM NaOH, 1.5 mM HCl and 0.1× PBS prior to mixing with growth factor reduced Matrigel (Becton Dickinson) to obtain a final collagen concentration of 1.4 mg/ml. Forty microliters of the Matrigel:Collagen mix was added to chamber slides and allowed to set at 37°C. Cells were seeded onto the Matrigel:Collagen bed at 4,000 cells/well in 3D Assay media [2% growth factor reduced Matrigel, 2% horse serum, 10 µg/ml insulin, 0.5 µg/ml hydrocortisone, 100 ng/ml cholera toxin, penicillin (100 U/ml) and streptomycin (100 U/ml)]. Assay media was replaced every 4 days and cultures were used for analysis at designated time points. Invasive acini were counted manually on day 8 with the average taken from 3 experiments using 2 independent cell lines. At least 3 fields were counted per condition.

SDS-PAGE and Western Blotting

Whole cell lysates were prepared in RIPA lysis buffer supplemented with the phosphatase inhibitor sodium vandate (100 mM) and Complete Mini Protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Protein concentrations were quantitated using Lowry Protein Assay (BioRad, Hercules, Calif., USA) and resolved on precast gradient gels (Invitrogen, Grand Island, N.Y., USA). Protein was transferred to PVDF membranes (Millipore, Billerica, Mass., USA) 30 V overnight at 4°C. Membranes were blocked in 2% milk/PBST for 1 h at room temperature. Membranes were incubated with primary antibodies in blocking solution overnight at 4°C [E-cadherin, BD No. 61081; phospho-mitogen-activated protein kinase kinase (pMEK) 41G9, Cell Signaling No. 9154; total MEK, Cell Signaling No. 4694; H-Ras MC57, Upstate No. 05-775; Scribble supernatant 7C6-D10; pSMAD2, AB3849 and α-tubulin clone B512, Sigma T5168]. Membranes were washed and incubated with appropriate HRP-conjugated secondary antibody at 1:5,000 for 45 min at room temperature. Membranes were thoroughly washed before developing using LumiLight (Roche) according to the manufacturer's instructions.

EGF and TGF β Stimulations

MCF10A cells were plated out at 7.5×10^4 cells in 6-well dishes. Twenty-four hours later culture media was replaced with EGF-depleted media. Cells were cultured in EGF-depleted media for 48 h before replacing with prewarmed media containing 20 ng/ml EGF for EGF stimulations or for 24 h before adding 20 ng/ml TGF β for TGF β stimulations. Cells were harvested for protein analysis at designated time points.

Soft Agar Culture

Subconfluent cells were resuspended at 6,000 cells/ml in media then mixed in a 1:1 ratio with 0.6% low melt agarose prewarmed to 37°C to obtain cells in media containing a final concentration of 0.3% agarose; 6,000 cells (2 ml) of the agarose/cell mix were plated onto a base layer of 0.7% agarose that had been allowed to set at room temperature. Cultures were incubated at 37°C for 14 days and replenished with fresh culture media every 4 days. Plates were stained using the viable cell indicator tetrazolium salt (Sigma, M2128), photographed and colonies counted using ImageJ software. Experiments were performed in triplicate and 4 experiments were carried out using 2 independent cell lines.

Staining of Cultures Grown in 2D

MCF10A cells were grown on sterile coverslips before fixing in 100% ice-cold methanol for 20 min. Coverslips were washed in PBS before blocking in 2% BSA/PBS for 1 h at room temperature. Goat anti-Scribble (C-20; Santa Cruz, sc-11049) diluted 1:200 in blocking buffer was applied to coverslips and incubated overnight at 4°C in a humid chamber. Coverslips were washed under agitation with PBST (0.05% Tween 20/PBS) before probing with Alexa-Fluor secondary antibody (Invitrogen) diluted at 1:1,000 for 1 h. Coverslips were washed with PBST before mounting with ProLong Gold Antifade containing DAPI (Invitrogen, P36935).

Staining of Cultures Grown in 3D

Immunofluorescent staining of MCF10A cells cultured in 3D was carried out essentially as previously described [Debnath et al., 2003]. Briefly, cultures were fixed in 3.7% paraformaldehyde for 30 min before permeabilization in 0.3% Triton X-100 for 10 min. Fixed cultures were washed with a glycine rinse (130 mM NaCl, 7 mM Na₂HPO₄, 3.5 mM NaH₂PO₄, 100 mM glycine) and blocked for 1 h in IF buffer (130 mM NaCl, 7 mM Na₂HPO₄, 3.5 mM NaH₂PO₄, 7.7 mM NaN₃, 0.1% BSA, 0.2% Triton X-100, 0.05% Tween 20) containing 10% horse serum. Cultures were incubated overnight at 4°C with primary antibodies [goat anti-Scribble (C-20), Santa Cruz No. sc-11049; mouse anti- β -catenin, Becton Dickinson No. 610153; Alexa-633 phalloidin, Invitrogen No. A22284] diluted in blocking buffer. Cultures were washed thoroughly with IF buffer before incubation with AlexaFluor secondary antibodies (Invitrogen) diluted at 1:1,000 for 1 h at room temperature. Cultures were washed before mounting in ProLong Gold Antifade with DAPI (Invitrogen, P36935).

Image Acquisition

All differential interference contrast images were taken on Leica Inverted microscope (Leica, Wetzlar, Germany) and all images of immunofluorescent staining were acquired on a BX51 scanning confocal microscope (Olympus, Mt. Waverley, Vic., Australia) using a 40 $\times$ oil objective.

Results

Generation and Characterization of Stable Wild-Type Scribble and Scrib P305L Mislocalization Mutant-Expressing MCF10A Lines

In order to study the function of the Scrib P305L mutation relative to Scrib wt, stable cell lines were generated to express pLPCX (vector control), human Scrib wt and the mislocalization mutant, Scrib P305L in MCF10As, an immortal yet untransformed mammary cell line. Increased Scribble expression in the Scrib wt and Scrib P305L lines was evident by Western blot (fig. 1a). Light microscopy of the cells showed that cell morphology was not obviously perturbed by expression of either the wild-type Scribble or the Scribble mutant (fig. 1b). Staining using an antibody directed to Scribble showed that vector control cells expressed endogenous Scribble only at the membrane, and in Scrib wt overexpressing cells, Scribble was correctly localized to the membrane with the high expression levels also resulting in cytoplasmic Scribble expression (fig. 1c). In the Scrib P305L overexpressing cells, Scribble was not localized to the membrane but rather showed strong cytoplasmic localization. This pattern of expression was also confirmed in 3D cultures (fig. 1d).

The Scrib P305L Mislocalization Mutation Alters Scribble's Ability to Regulate EGF and TGF β -Dependent MAPK-ERK Signaling

We have previously shown that ectopic expression of wild-type Scribble suppresses MAPK-ERK signaling in the context of oncogenic Ras^{V12} [Dow et al., 2008]. Here we investigated whether ectopic expression of either the wild-type Scribble or the Scrib P305L mutant was sufficient to suppress MAPK-ERK signaling in the context of wild-type Ras. In contrast to wild-type Scribble, the Scrib P305L mutant did not suppress phosphorylation of MEK as measured by the presence of pMEK in response to EGF stimulation and instead showed a sustained activation pattern compared to the vector control (fig. 2a). This function of Scribble is not EGF specific as expression of Scrib wt could also suppress the MAPK-ERK pathway upon activation of TGF β signaling and Scrib P305L could not (fig. 2b). Activation of MAPK-ERK signaling upon TGF β stimulation is necessary for TGF β -induced epithelial to mesenchymal transition (EMT) [Xie et al., 2004; Xu et al., 2009]. Scrib wt or Scrib P305L cells did not show suppression of the classical TGF β pathway (the SMAD pathway) as illustrated by the consistent expression pattern of phosphorylated SMAD2, a core TGF β transcrip-

Fig. 1. Characterization of the Scrib P305L mutant cells. **a** Western blot showing Scribble expression in pLPCX, Scrib wt and Scrib P305L stable cell lines. **b** Representative differential interference contrast images of indicated cell lines. **c** Confocal images of indicated cells cultured in 2D and stained with anti-Scribble antibody

to verify mislocalization of Scribble in the Scrib P305L mutant cells. **d** Confocal images of indicated cells cultured in 3D and stained with anti-Scribble antibody to verify mislocalization of Scribble in the Scrib P305L mutant cells.

tional regulator, across the groups (fig. 2b). These data suggest that wild-type Scribble but not the mislocalized P305L Scribble mutant may suppress both EGF and TGF β -induced EMT phenotypes and that localization of Scribble could be critical for this function.

A Mutation That Causes Scribble Mislocalization Affects Restoration of Ras^{V12}-Mediated E-Cadherin Loss

To examine how the changes in MAPK-ERK signaling kinetics that are a consequence of alterations in Scribble expression and localization impact on biological processes, and in particular cancer-related processes such as EMT, we generated stable MCF10A cell lines similar to those described above but in the presence or absence of oncogenic H-Ras^{V12} (fig. 3). The classical Ras proteins (H-, K- and N-Ras) have been well established as key players in many cancer-related processes including pro-

liferation, apoptosis, differentiation and adhesion [Mallumbres and Barbacid, 2003; Pylayeva-Gupta et al., 2011]. In this study, we examined the effect of Scribble in universally used assays of Ras^{V12}-induced transformation.

E-cadherin loss is associated with dissolution of adherens junctions and is frequently considered to be a hallmark of EMT, part of a process that encompasses loss of apico-basal polarity, cytoskeletal rearrangements and changes in the extracellular matrix, migration and invasion [Xu et al., 2009; Godde et al., 2010; Savagner, 2010]. Immunofluorescence staining in pLPCX-MSCV, Scrib wt-MSCV and Scrib P305L-MSCV cells, showed E-cadherin to be localized to the cell surface at adhesion junctions, indicating that expression of either Scrib wt or Scrib P305L does not alter E-cadherin localization (data not shown). We then looked at expression levels of E-cadherin in the context of Ras^{V12} by Western blot. As expected, we saw a dramatic reduction in E-cadherin expression

Fig. 2. Scrib wt suppresses MAPK-ERK signaling yet Scrib P305L does not. **a** Western blot on protein lysates harvested from pLPCX, Scrib wt and Scrib P305L cells probed for the MAPK-ERK components pMEK and total MEK following EGF stimulation (i), and quantification of pMEK levels relative to total MEK (ii). **b** Immunoblot on indicated protein lysates probed for pMEK, total MEK, pSMAD2, Scribble and α-tubulin following TGFβ stimulation.

in the pLPCX-Ras^{V12} cell lines (fig. 3). Consistent with what we have previously reported, overexpression of wild-type Scribble in the presence of oncogenic Ras largely restores E-cadherin [Dow et al., 2008] (fig. 3). However, despite similar expression levels of Scribble in both the Scrib wt and the Scrib P305L cells (fig. 1a), expression of Scrib P305L did not show a rescue of E-cadherin expression to the same extent as wild-type Scribble (fig. 3). Therefore, a mutation that causes mislocalization of Scribble from the cortex to the cytoplasm reduces the ability of Scribble to restore E-cadherin in the context of oncogenic Ras signaling.

The Scrib P305L Mislocalization Mutant Is Unable to Suppress Ras^{V12}-Induced Invasion

The ability of cells to invade through the extracellular matrix is another common phenomena associated with tumorigenesis and EMT. To assess the effect of wild-type

versus mislocalized Scribble, we cultured Scrib wt and Scrib P305L cells in 3D using a Matrigel:Collagen matrix to determine invasive potential. A strong suppression of Ras^{V12}-dependent invasion was seen 8 days after seeding when wild-type Scribble was expressed (fig. 4a) [Dow et al., 2008]; however, Scrib P305L-Ras^{V12} cells exhibited nominal suppression and acini commonly presented with invasive spikes similar to those seen in pLPCX-Ras^{V12} cells (fig. 4a). From this data, we conclude that the P305L mutation that causes Scribble mislocalization also disrupts Scribble's ability to suppress the invasive phenotype of Ras^{V12} cells. To visualize the morphology of the acini and to ensure that Ras^{V12} expression did not alter the localization of Scribble previously described and shown in figure 1, cultures were fixed and stained at day 16. Scribble retained the cytoplasmic rather than normal membrane-bound localization in the Scrib P305L-expressing cells (fig. 4b). Wild-type MCF10A cells formed well-de-

Fig. 3. Scrib wt restores E-cadherin expression more effectively than Scrib P305L in the context of oncogenic Ras^{V12} expression. Western blot showing Scribble, Ras and E-cadherin expression in (pLPCX, Scrib wt and Scrib P305L) ± Ras^{V12} stable cell lines.

veloped hollow acini by day 16 analogous to those seen in the pLPCX-MSCV vector control and in the Scrib wt-MSCV cells. A larger number of apoptotic nuclei (detected by DAPI staining) were seen in the lumen of acini expressing Scrib P305L (fig. 4b), consistent with previous reports of a Scrib P305L-dependent delay in clearing of the lumen [Zhan et al., 2008].

To more closely examine the morphology of these cells we performed 3D Matrigel:Collagen assays and stained for the adherens junction marker and Wnt signaling activator β -catenin and the cytoskeletal component F-actin (fig. 5). In all cells β -catenin was detected only at the membrane indicating intact adherens junctions with the exception of the pLPCX-Ras^{V12} cells where β -catenin expression was reduced and diffuse. This is consistent with previous reports where expression of Scrib P305L in MDCK cells did not perturb membrane-associated β -catenin [Legouis et al., 2003]. We used phalloidin to visualize F-actin and, although we saw a reduction in cortical actin in the lines expressing Ras^{V12}, there were no notable differences between the Scrib wt-Ras^{V12} and the Scrib P305L-Ras^{V12} cells suggesting that mislocalization of Scribble does not markedly alter the structure of the cytoskeleton (fig. 5). Ras^{V12} expression results in less polarized acini [Dow et al., 2008] and a reduction in β -catenin and F-actin on luminal surfaces. Wild-type Scribble restores expression and localization of β -catenin and F-actin in Ras^{V12} expressing cells compared to the pLPCX-MSCV control cells whilst the presence of the P305L mutant results in a less potent restoration in this context (fig. 5).

Suppression of Ras^{V12}-Induced Anchorage-Independent Cell Growth by Scribble Is Independent of Scribble Localization

Anchorage-independent cell growth is considered one of the hallmarks of cell transformation and is commonly used as an assay to assess the malignant transformation of cells. To assess how the expression of the Scrib P305L mislocalization mutant affects this compared to wild-type Scribble, we grew cell lines in soft agar for 14 days and measured colony formation. We found the presence of Ras^{V12} was required for anchorage-independent growth, as pLPCX-MSCV, Scrib wt-MSCV and Scrib P305L-MSCV cell lines did not form colonies (data not shown). Surprisingly, when analyzed relative to the colony number in pLPCX-Ras^{V12} cells, both Scrib wt-Ras^{V12} and Scrib P305L-Ras^{V12} cells showed a significant and similar suppression in colony formation in soft agar (fig. 6). This data indicates that Scribble localization is not crucial to the pathways used in the suppression of Ras^{V12}-induced anchorage-independent cell growth.

Discussion

Loss of Scribble has been reported in both *Drosophila* and mammalian settings to cooperate with oncogenes and promote cancer progression [Brumby and Richardson, 2003; Pagliarini and Xu, 2003; Dow et al., 2008; Zhan et al., 2008; Pearson et al., 2011]. We have previously shown that overexpression of Scribble can suppress transformation and invasion in Ras^{V12}-expressing MCF10A cells [Dow et al., 2008]. Here we show that not only does Scribble function as a tumor suppressor in this setting but Scribble can also suppress MAPK-ERK signaling in the absence of activated Ras. As there have been several reports in the literature of Scribble mislocalization in human cancers, we examined how a mislocalization mutant of Scribble alters cancer-associated phenotypes. We found that localization of Scribble away from the membrane phenocopies Scribble loss-of-function for assays of MAPK-ERK signaling, Ras^{V12}-induced invasion and Ras^{V12}-induced E-cadherin loss. Importantly, mislocalization of Scribble did not significantly change the ability to suppress Ras^{V12}-dependent anchorage-independent growth. This finding illustrates that mislocalization in a cancer setting may contribute to some yet not all tumor-promoting properties.

It was somewhat surprising to find that Scrib P305L, which shows localization away from the membrane, was able to suppress colony formation in soft agar (fig. 6) and

Fig. 4. Scrib wt suppresses Ras^{V12}-induced invasive growth in Matrigel:Collagen, but Scrib P305L does not. **a** Differential interference contrast images of indicated cell lines cultured in Matrigel:Collagen for 8 days (i) and quantification of the percent-

age of invasive acini in each group (ii). Error bars represent SD; n = 3; p values were calculated using paired Student's t test. **b** Confocal images of indicated cell lines cultured in Matrigel:Collagen for 16 days and stained for Scribble and the DNA marker, DAPI.

therefore retain this tumor suppressive function. Not much is known about the features that a cell must carry in order to display anchorage-independent growth and what pathways are involved. One study investigating the mechanisms behind anchorage-independent growth found the most important pathway to be the phosphati-

dylinositol 3-kinase (PI3K)/Akt pathway and that functional activation of signal transducer and activator of transcription-3 partially overlaps with PI3K/Akt in promoting anchorage-independent growth [Wang, 2004]. The same study also proposed that Cdc42, and to some extent Rac1, play a role in promoting anchorage-inde-

Fig. 5. Mislocalization of Scribble does not alter the localization of β -catenin. Confocal images of indicated cell lines cultured in Matrigel:Collagen for 16 days and stained for Scribble, the DNA marker DAPI, the adherens junction and Wnt signal activator β -catenin, and the cytoskeletal component F-actin.

pendent growth. Other arms of MAPK signaling have been implicated to play regulatory roles in anchorage-independent cell growth such as the pp38 pathway [Tadlock and Patel, 2001].

Erbin is another member of the LAP family of proteins and has similarities to Scribble. Structural analysis has predicted that the LRR domain of Erbin forms a horse-shoe-like structure with parallel β -sheets on the inner circumference and α -helices flanking the outer sides [Kajava and Kobe, 2002]. Crystal structure modeling performed by Legouis et al. [2003] of the LRR domains in the *C. elegans* homolog of Scribble revealed a similar structure and predicts that the P305L mutation affects the helicoidal part of the thirteenth LRR and, in turn, the binding potential of the entire domain. LAP proteins are considered

scaffolding proteins and have been proposed to mediate signal transduction and organize molecules into discrete subcellular localizations, thus facilitating physical or genetic interactions. The LAP protein Erbin exhibits these characteristics and, interestingly, the LRR domain has been shown to interact with Ras and dampen MAPK-ERK signaling [Dai et al., 2007; Dan et al., 2010]. Furthermore, the *Xenopus* SUR-8 protein, which contains an LRR domain similar to LAP proteins such as Scribble but does not localize to the membrane, interacts with Ras proteins and modulates downstream signaling [Sieburth et al., 1998]. Scribble also acts to mediate signaling pathways and interacts with MAPK signaling components including ERK [Dow et al., 2008; Humbert et al., 2008; Zhan et al., 2008; Nagasaka et al., 2010]. As reported here,

Fig. 6. Both Scrib wt and Scrib P305L cells suppress Ras^{V12}-dependent anchorage-independent growth. Quantification of the colony number in Ras^{V12} cell lines is shown along with representative images of colony growth. Error bars represent SD; n = 4; p values were calculated using paired Student's t test.

Scribble mislocalization interferes with the normal function in signaling transduction, resulting in changes to the tumorigenic phenotype. It must be noted that the structural changes likely to result from the P305L mutation could notionally affect the binding of proteins that are crucial for suppression of MAPK-ERK signaling and invasion yet not anchorage-independent growth. As Scribble is a scaffolding protein, it is probable that a point mu-

tation affecting localization will also affect binding domains critical for its function. Mislocalization and abnormal levels of cytosolic Scribble may thus be additional to structural changes and further studies are required in order to discern direct links between mislocalization and its functional consequence. Interestingly, Scribble mislocalization from the cortex to the cytoplasm has also been demonstrated for a point mutation (R1535Q) in the C-terminus of Scribble which was identified in a human patient presenting with craniorachischisis, a severe neural tube defect [Robinson et al., 2012]. As it is less likely that a mutation in this region would dramatically disrupt Scribble structure and as functional roles for the C-terminal have yet to be determined, it would be informative to perform similar assays alongside those described here using a Scribble R1535Q construct to more specifically determine the direct role mislocalization has on Scribble function.

Overall, the work presented here has examined two different tumor promotion-related properties, invasion and anchorage-independent growth; we show modulation of these properties by the polarity protein Scribble. We report that these properties are differentially sensitive to the localization of Scribble. We propose that such differential functional consequences of protein mislocalization may apply to other proteins and, therefore, care must be taken when assessing and interpreting how altered protein characteristics such as mislocalization contribute to individual pathways and cellular properties that drive tumorigenesis.

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