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ORIGINAL ARTICLE

The cell polarity regulator hScrib controls ERK activation through a KIM site-dependent interaction

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The cell polarity regulator, human Scribble (hScrib), is a potential tumour suppressor whose loss is a frequent event in late-stage cancer development. Little is yet known about the mode of action of hScrib, although recent reports suggest its role in the regulation of cell signalling. In this study we show that hScrib is a direct regulator of extracellular signal-regulated kinase (ERK). In human keratinocytes, loss of hScrib results in elevated phospho-ERK levels and concomitant increased nuclear translocation of phospho-ERK. We also show that hScrib interacts with ERK through two well-conserved kinase interaction motif (KIM) docking sites, both of which are also required for ERK-induced phosphorylation of hScrib on two distinct residues. Although wild-type hScrib can down-regulate activation of ERK and oncogenic Ras co-transforming activity, an hScrib mutant that lacks the carboxy terminal KIM docking site has no such effects. These results provide a clear mechanistic explanation of how hScrib can regulate ERK signalling and begin to explain how loss of hScrib during cancer development can contribute to disease progression.
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Introduction

The mitogen-activated protein kinase (MAPK) pathways that activate extracellular signal-regulated kinase (ERK), c-Jun amino-terminal kinase (JNK) and p38 kinases have important roles in modifying the morphogenetic and motile responses of cells. Among these pathways, the Ras/Raf/MEK/ERK signal transduction cascade is a key mechanism for regulating cell fate in response to growth, proliferation, differentiation and survival signals (Fang and Richardson, 2005; Kolch, 2005; Torii *et al*., 2006; Yoon and Seger, 2006). Activation of the cascade ultimately results in the activation of ERK and its dissociation from the MEK–ERK complex, which then stimulates gene expression, cytoskeletal rearrangements and cell metabolism, coordinating the cell's responses to a variety of extracellular signals (Schaeffer and Weber, 1999; Fincham *et al*., 2000). Aberrations in ERK1/2 signalling are also known to be involved in a wide range of pathologies, including many cancers, diabetes, viral infections and cardiovascular disease. This pathway is hyperactivated in many tumours, with activating mutations of Ras occurring in approximately 15–30% of all human cancers (Malumbres and Barbacid, 2003; Garnett *et al*., 2005).

Recent studies have shown that proteins involved in the regulation of cell polarity can also affect cell signalling cascades. Two of the most well-characterized of these proteins are human discs large (hDlg) and human Scribble (hScrib). In *Drosophila*, these proteins cooperate to regulate pathways of cell polarity and cell proliferation control (Bilder *et al*., 2000; Bilder, 2004; Zeitler *et al*., 2004). In humans, the function of these proteins is less clear. However, both are targets for several human tumour viruses and the expression of both hDlg and hScrib is frequently lost during the later stages of malignant progression, suggesting that they possess potential tumour suppressor functions in human cells (Kiyono *et al*., 1997; Gardiol *et al*., 1999, 2006; Nakagawa and Huibregtse, 2000; Nakagawa *et al*., 2004; Navarro *et al*., 2005; Nagasaka *et al*., 2006). In the case of hDlg, multiple phosphorylation events by p38γ and JNK have been shown to regulate its localization (Sabio *et al*., 2005; Massimi *et al*., 2006) and recent studies have also shown that the entire hScrib cell polarity complex, comprising hDlg, hScrib and Hugl1 (human lethal giant larvae), is dynamically regulated after activation of the MAPK signalling cascade (Massimi *et al*., 2008). A more direct effect of hScrib on the regulation of this cascade has also been shown. In one study, hScrib was shown to be able to inhibit signalling downstream of Ras and Raf, but upstream of ERK (Dow *et al*., 2008), with loss of hScrib enhancing Ras-induced cell invasion. In a separate study, hScrib was also shown to be involved in regulating oncogene-induced apoptosis in a JNK-dependent manner, with loss of Scribble cooperating with c-myc in a mouse model of mammary carcinogenesis (Zhan *et al*., 2008).

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All of the above data indicate that Scribble can act by modulating MAPK signalling and show a clear role for Scribble as a suppressor of tumour invasion. However, in the context of the Ras/Raf/MEK/ERK signalling module, there is no information as to how this is inhibited by Scribble. In this study, we now show that hScrib downregulates ERK activation and inhibits nuclear translocation of activated ERK through a direct protein–protein interaction, thereby providing a direct mechanism for hScrib regulation of the ERK signalling cascade.

Results

Loss of hScrib enhances ERK nuclear localization

Previous studies have shown that loss of hScrib cooperates with activated ras in the induction of invasion in MCF10A cells, an activity that seemed to be related with Scribble's ability to downregulate ERK signalling (Dow *et al*., 2008). We therefore first wanted to investigate whether hScrib could potentially affect ERK signalling in human keratinocytes. To do this, we generated a series of HaCaT cell lines in which hScrib levels had been ablated using short hairpin RNA targeting vectors (Massimi *et al*., 2008). Cells were then either left untreated or exposed to osmotic shock for 30 min to enhance ERK activation. The cells were then extracted and the levels of hScrib, total ERK and activated phospho-ERK were monitored by western blotting. The results in Figure 1a show a modest level of constitutively active ERK in HaCaT cells. However, in the hScrib knockdown cells there is a marked increase in the levels of phospho-ERK in both cell lines and this increases further after osmotic shock. These results show that hScrib can contribute to the regulation of the ERK signalling cascade in human keratinocytes.

Activated ERK has been shown to translocate to the nucleus (Chen *et al*., 1992; Gonzalez *et al*., 1993; Lenormand *et al*., 1993; Treisman, 1996; Fukuda *et al*., 1997; Khokhlatchev *et al*., 1998; Pouyssegur *et al*., 2002). We therefore investigated whether there was a change in the pattern of ERK localization in HaCaT cells when hScrib expression levels were reduced. To do this, immunofluorescence analysis of total and phospho-ERK expression was carried out on the control and shScrib cell lines, and the results obtained are shown in Figure 1b. As can be seen, loss of hScrib (Figure 1biii) also results in a significant increase in the amount of nuclear-translocated ERK. In contrast, activated ERK seems to accumulate in Golgi-like structures in the cytoplasm of the control cells (Figures 1bi and bii), consistent with previous reports (Torii *et al*., 2004). To verify these results, we also performed a series of transient small interfering RNA experiments, in which hScrib levels were ablated in HEK293 cells, and the levels of phospho-ERK in both total cell extracts (Figure 2a) or in the respective cellular fractions (Figure 2b) were analysed by western blotting. In both cases, loss of hScrib enhanced ERK activation and also resulted in enhanced nuclear accumulation of active phospho-ERK. These results show that one consequence of hScrib knockdown is enhanced nuclear translocation of activated ERK.

hScrib is a substrate of ERK and PKA

Having confirmed that hScrib could regulate ERK activation and nuclear translocation, we next wanted to investigate the mechanism by which this might occur. Analysis of the hScrib sequence revealed the presence of two perfect ERK-binding sites (kinase interaction motif (KIM) sites) at positions 836aa–846aa and 1396aa–1404aa (Figure 3). In addition, two potential ERK phospho-acceptor sites are correspondingly located at residues S853 and S1448, downstream of each of the two KIM sites (Figure 3).

To first investigate whether either of these two potential phospho-acceptor sites on hScrib was phosphorylated *in vivo*, we transfected cells with hemagglutinin (HA)-tagged hScrib expression plasmid and grew the cells with or without osmotic shock. The cells were then extracted, and hScrib protein was immunoprecipitated with anti-HA agarose beads. The subsequently gel-purified protein was then subjected to mass spectroscopy analysis. A summary of the phospho-peptides that were identified under the two culture conditions is shown in Figure 3. As can be seen, the N-terminal site at position S853 is phosphorylated in unstressed conditions, as is S1445 in the carboxy terminal region of the protein. Interestingly, after exposure to osmotic shock, S853 remains phosphorylated, whereas S1445 is no longer phosphorylated and the phosphorylation event occurs exclusively on S1448, just three amino acids downstream. These results show clear differential phosphorylation of hScrib *in vivo*, both before and after osmotic stress.

On the basis of these data we reasoned that ERK was a prime candidate kinase for the phosphorylation events at S853 and S1448, with the corresponding upstream KIM sites located approximately at residues 836 and 1396, respectively, whereas the phospho-site at S1445 corresponds to a potential PKA recognition site. To first confirm whether these were the responsible kinases, we analysed whether hScrib was a substrate for ERK and PKA *in vitro*. To do this, a glutathione *S*-transferase (GST)–hScrib fusion protein was purified and incubated with the purified recombinant kinases and [γ^{32}P]-ATP, and the results obtained are shown in Figure 4a. As can be seen, GST–hScrib is a good substrate for phosphorylation by both PKA and ERK1, and is only a very weak substrate for JNK and ERK2. To then determine whether the putative KIM and phospho-acceptor sites on hScrib corresponded to those identified *in vivo*, we generated a series of GST–hScrib fusion proteins that had been mutated in both the ERK KIM recognition sites, the two potential ERK phospho-acceptor sites and in the potential PKA phospho-acceptor site (Figure 4b). The purified GST proteins were then incubated with the purified kinases and [γ^{32}P]-ATP, and the results obtained are shown in Figure 4c. As can be seen, the PKA phospho-acceptor site on

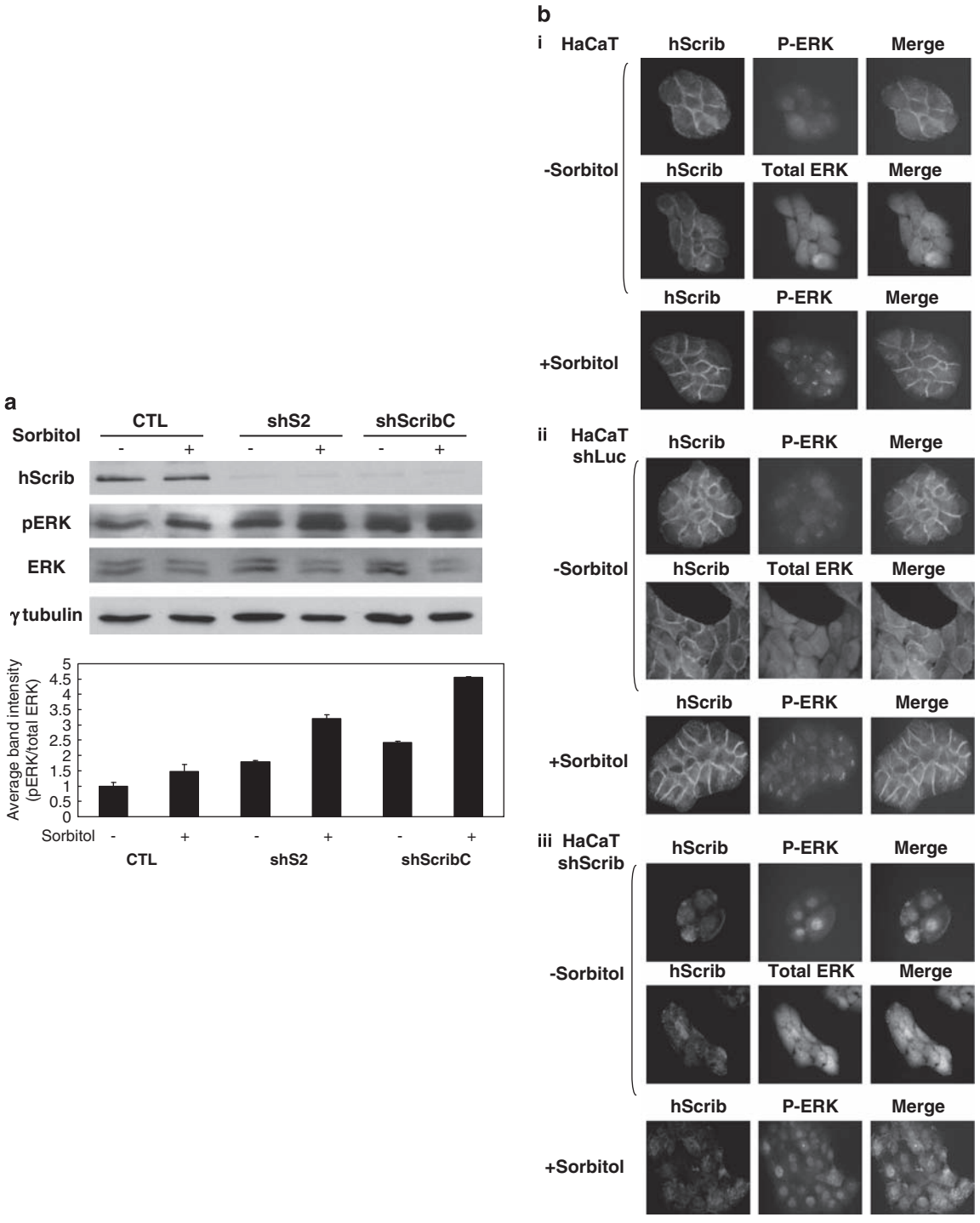

Figure 1 hScrib suppresses/downregulates the Raf/MEK/ERK pathway. (**a**) The sh-hScrib stable cell lines (S2, ScribC) and control cells (CTL) were cultured overnight and either left untreated or exposed to sorbitol for 30 min as indicated. The cells were then harvested and levels of ERK, phospho-ERK and hScrib were analysed by western blotting. γ-Tubulin was used as a loading control. The lower histogram shows the quantitative analyses of the intensities of the pERK bands from three independent experiments with s.d. indicated. (**b**) Immunofluorescent analysis of hScrib and ERK expression. HaCaT cells (i), sh-Luc control TR cells (ii) and sh-hScrib cells (iii) were grown on coverslips and then exposed to sorbitol as indicated. The cells were then fixed and double stained with the anti-hScrib antibody, the anti-phospho-ERK1/2 antibody or the anti-total ERK1/2 antibody.

GST–hScrib maps precisely to residue S1445 identified in the mass spectroscopic analysis. In the case of ERK1 the results are more complex. First, there are clearly two phospho-acceptor sites, at S853 and S1448, again corresponding to the two sites that were identified *in vivo*. Both KIM sites seem to be important for ERK1 recognition, with mutation of either site decreasing the phosphorylation to a level equivalent to that observed

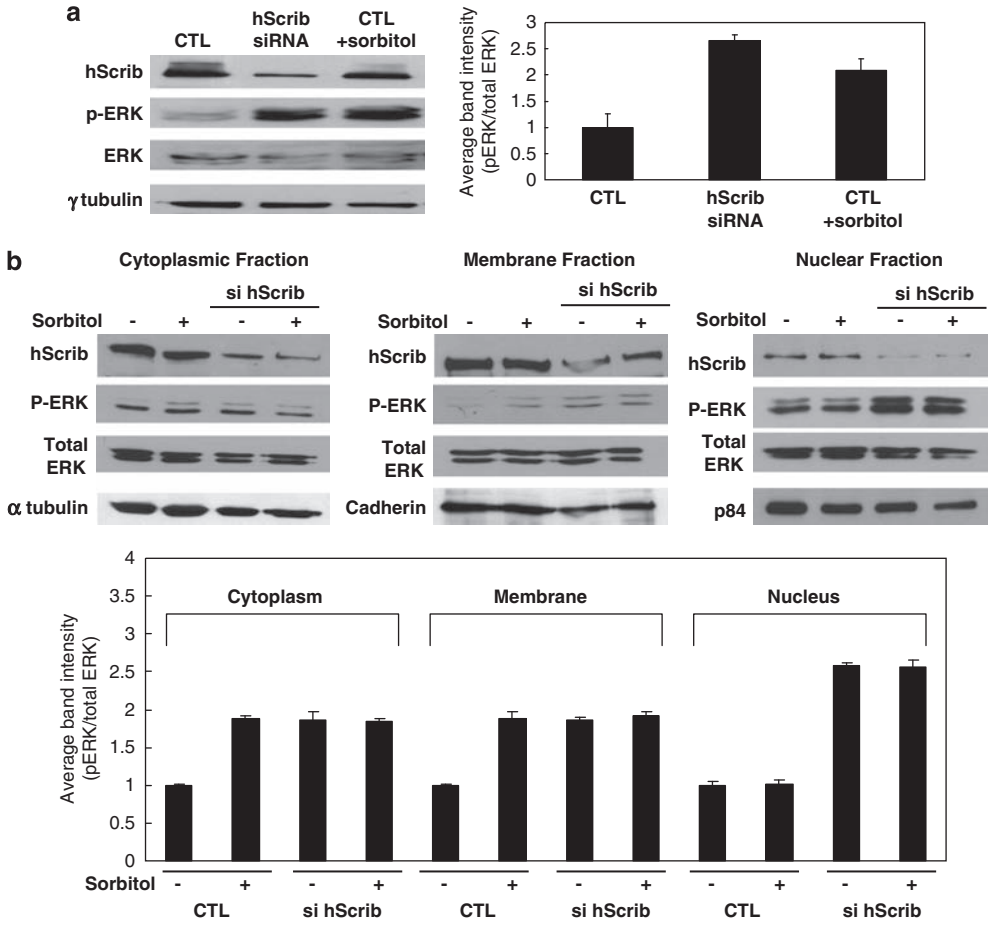

Figure 2 Loss of hScrib enhances phospho-ERK nuclear translocation. (**a**) HEK293 cells were transfected with hScrib siRNA and siLuc RNA as control (CTL). Total cell extracts were then made after 48 h, and hScrib, pERK, ERK and γ-tubulin were detected by western blotting. The right-hand histogram shows the quantitative changes in phospho-ERK/total ERK levels from a minimum of three independent assays. (**b**) HEK293 cells were transfected with hScrib siRNA and siLuc RNA as control, and then exposed to sorbitol for 20 min as indicated. Cells were fractionated into cytoplasmic, membrane and nuclear pools then phospho-ERK was then detected by western blotting. p84 was used as a loading control for the nuclear fraction, E-cadherin was used as a loading control for the membrane fraction and α-tubulin was used as the loading control for the cytoplasmic fraction. The lower histogram shows the quantitative changes in phospho-ERK/total ERK levels from a minimum of three independent assays. Note the relative increase in nuclear phospho-ERK after hScrib knockdown.

with the respective single phospho-site mutations. Most importantly, the double KIM site mutations, or the double phospho-acceptor site mutations, completely abolish ERK phosphorylation of hScrib (Figure 4c). In contrast, all of these mutants are still recognized by PKA (Figure 4d), showing that these mutations do not overly perturb the overall structure of hScrib, and further show the specificity of the assays. These results show that hScrib has two ERK docking sites and two corresponding phospho-acceptor sites, with S853 phosphorylated under normal growth conditions and S1448 being phosphorylated under conditions of osmotic stress.

hScrib regulates ERK activation through the two KIM docking sites

We next wanted to investigate whether the two identified KIM sites could actually serve as docking sites for ERK *in vitro* and *in vivo*. The GST–hScrib fusion proteins were first used in pull-down assays using the commercially purified ERK1, and levels of bound ERK1 were assessed by western blotting. The results obtained are shown in Figure 5a and show a strong direct interaction between the wild-type hScrib and ERK1. In contrast, mutation of the C-terminal KIM site largely abolishes the interaction, showing that most of the interaction is through this carboxy terminal site, although a weaker interaction is also mediated by the N-terminal KIM site. To investigate whether these sites on hScrib were also responsible for ERK binding *in vivo*, we first used the GST–hScrib fusion proteins to pull down ERK from cell extracts and then analysed by western blotting for total and phospho-ERK. The results obtained are shown in Figure 5b. As can be seen, the two KIM sites contribute to hScrib binding to ERK, although the C-terminal site seems to be the strongest site of interaction, with mutation of this single site almost abolishing ERK

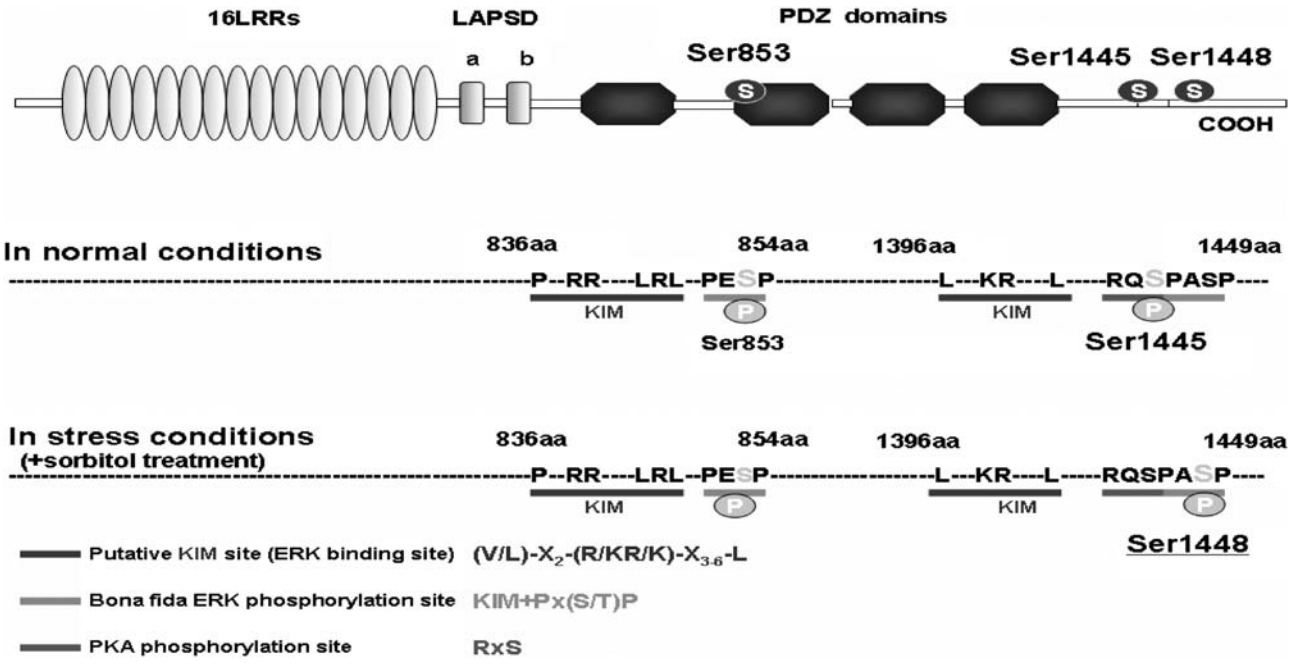

Figure 3 Identification of hScrib phospho-acceptor sites. Lysates from HEK293 cells transfected with HA-tagged hScrib either in the absence (−) or presence (+) of sorbitol for 30 min were subjected to immunoprecipitation with an anti-HA antibody. Complexes were run on SDS–PAGE and the hScrib gel slice was then subjected to mass spectrometry. Residues at S853, S1445 and S1448 were identified as phospho sites. The consensus ERK-phosphorylation motif (PESP, PASP), the consensus PKA-phosphorylation motif (RXS) (Pearson and Kemp, 1991) and the putative ERK docking site or a kinase interaction motif ((V/L)-X_2-(R/K)-(R/K)-X_{3-6}-L) (MacKenzie *et al*., 2000; Tanoue *et al*., 2000; Fantz *et al*., 2001; Zhou *et al*., 2006) are shown, in which X is any amino acid.

binding. Interestingly, when extracts are made from cells exposed to osmotic stress, there is a marked increase in the amount of ERK complexed with hScrib, suggesting that ERK activation can enhance its interaction with hScrib. We also carried out co-immunoprecipitation experiments in which HA-tagged wild-type and the ΔKIM mutant of hScrib were transfected into HEK293 cells, and then immunoprecipitated with anti-HA agarose beads. The co-precipitated ERK was then detected by western blotting. The results in Figure 5c also show that hScrib binds ERK *in vivo* in a KIM site-dependent manner and, in addition, this interaction also seems to be enhanced when the cells are exposed to osmotic stress.

The above results show that loss of hScrib enhances ERK activation and that hScrib can interact with ERK through two KIM docking sites. We next wanted to investigate whether the ability of hScrib to directly interact with ERK was responsible for its ability to downregulate ERK activation. To do this, HEK293 cells were transfected with the wild-type hScrib and the ΔKIM mutants. After 24 h, the cells were extracted and the levels of activated phospho-ERK were analysed by western blotting. The results obtained are shown in Figure 6 and show a clear downregulation in the levels of phospho-ERK when wild-type hScrib is overexpressed. In contrast, the hScrib that has the carboxy terminal KIM site mutation is no longer capable of affecting the levels of ERK phosphorylation, whereas the amino terminal KIM site mutant can downregulate phospho-ERK to levels close to those obtained with the wild-type hScrib. These results show that hScrib can directly regulate the levels of ERK activation through a direct protein–protein interaction with ERK.

To determine whether hScrib downregulation of ERK activation was physiologically relevant, we analysed the effects of the wild-type and non-ERK binding mutant of hScrib in an oncogene cooperation assay. Primary baby rat kidney (BRK) cells were transfected with human papillomavirus-16 E7 plus EJ-ras, in the presence or absence of the hScrib-expressing plasmids. After 3 weeks, the cells were fixed and stained and the numbers of colonies counted. The results obtained are shown in Figure 7 and Table 1. As can be seen, wild-type hScrib can inhibit the oncogene cooperation between E7 and EJ-ras, whereas a non-ERK binding mutant of hScrib is compromised in this activity. These results show that hScrib binding to ERK is functionally relevant in an assay of oncogene cooperation.

Discussion

Previous studies have reported many diverse functions for the hScrib protein. These include regulation of cell proliferation, cell polarity, cell migration and cell invasion in a variety of different cell types. Perhaps the most intriguing of these observations are the demonstrations that hScrib can potentially regulate MAPK signalling. Loss of hScrib was reported to enhance cell survival by inhibiting JNK-induced apoptosis in mammary tumour models of oncogene-induced

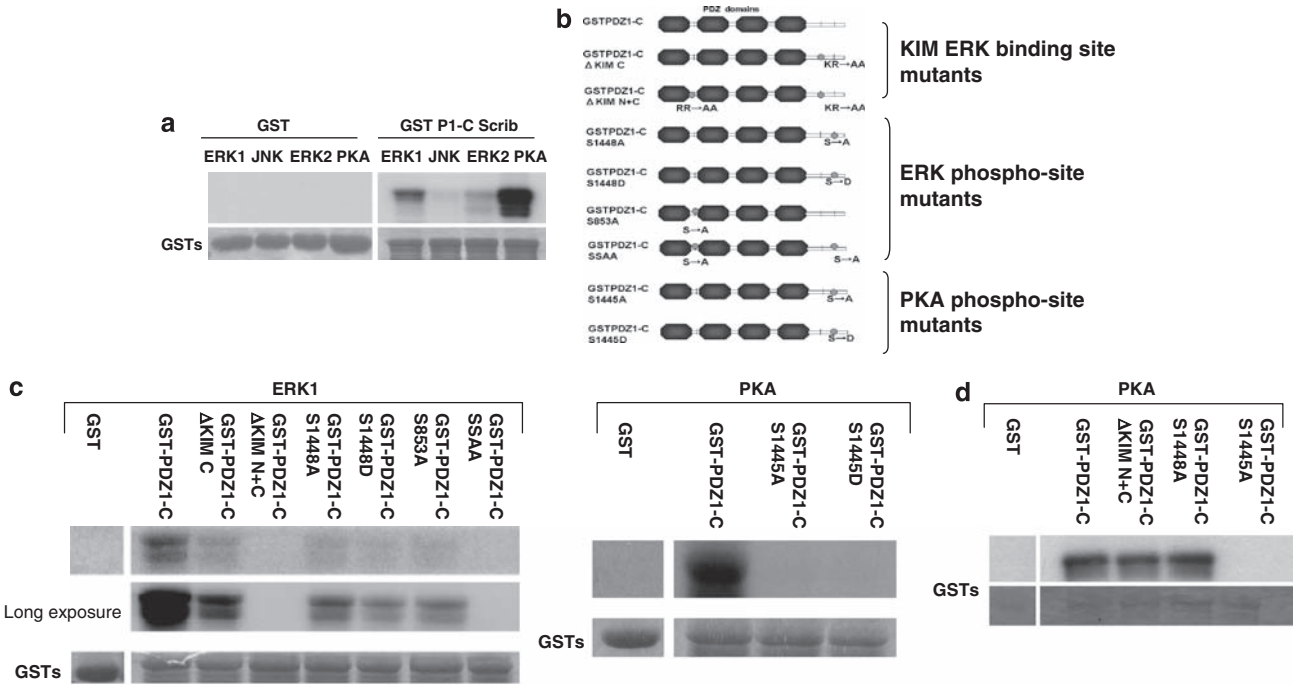

Figure 4 hScrib is a substrate for ERK1 and PKA. (**a**) The GST–hScrib fusion protein PDZ1-C (P1-C hScrib) and GST alone were incubated with purified ERK1, ERK2, JNK and PKA together with radiolabel, and after 20 min the level of phosphorylation was ascertained by SDS–PAGE and autoradiography (upper panels). The lower panels show the Coomassie protein stain of the gels. (**b**) A panel of hScrib mutants in which the ERK consensus phospho-acceptor sites (1448 and 853) and the PKA phospho-acceptor site (1445) were substituted with alanine or aspartic acid either individually or in combination. The ERK docking KIM sites (836 and 1396) sites were similarly either singly KR/AA (C terminal; ΔKIM C) or doubly RR/AA:KR/AA (N-terminal and C-terminal; ΔKIM N + C) mutated. (**c**) The wild-type and mutant hScrib fusion proteins with GST as control, were subjected to *in vitro* phosphorylation assays with ERK1 (left) or PKA (right) and then analysed by SDS–PAGE and autoradiography. The bottom panels show the Coomassie protein stains of the gels. (**d**) The wild-type and mutant hScrib fusion proteins defective in ERK1 recognition were subjected to *in vitro* phosphorylation assays with PKA and then analysed by SDS–PAGE and autoradiography. The bottom panel shows the Coomassie protein stain of the gel.

carcinogenesis (Zhan *et al*., 2008). hScrib was also reported to act upstream of ERK in the Ras/Raf/MEK/ERK signalling cascade to inhibit ERK activation and suppress Ras-induced cell invasion in breast epithelial cells (Dow *et al*., 2008). In this study, we show that hScrib regulates the ERK signalling pathway in human keratinocytes through a direct protein interaction with ERK. The consequences of this are inhibition of ERK phosphorylation and subsequent inhibition of ERK nuclear translocation.

In human skin keratinocytes, we observed that loss of hScrib expression induces an upregulation in the levels of activated phospho-ERK, providing the first indication that hScrib might also regulate ERK signalling in these cells. Most interestingly, loss of hScrib expression is accompanied by a marked accumulation of active phospho-ERK in the Golgi apparatus and in the nucleus. Although nuclear localized ERK is most likely involved in the regulation of gene expression related to cell cycle progression, Golgi accumulation may be related to the control of cell survival and cell migration, both of which have also been shown to be regulated by hScrib (Qin *et al*., 2005; Dow *et al*., 2008; Nola *et al*., 2008).

An understanding of how hScrib directly regulates ERK function has come from the identification of two ERK docking sites on hScrib. These two KIM sites are found at N- and C-terminal locations on hScrib, and both are essential for directing the interaction between ERK and hScrib, but with the C-terminal site having the strongest affinity for ERK. One of the most likely consequences of this interaction is to inhibit ERK translocation to the nucleus. However, an additional important feature is the direct inhibition of ERK activation as a result of the ability of hScrib to bind ERK. The mechanism by which this is achieved remains to be determined, although recruitment of de-activating phosphatases to the complex remains an intriguing possibility.

During the course of this analysis, we mapped three phospho-acceptor sites on hScrib. Under normal growth conditions, hScrib is phosphorylated at S853, most likely by ERK, and at S1445 by PKA. Interestingly, stimulation of MAPK by osmotic stress results in a marked loss of phosphorylation at the PKA site S1445, but a concomitant increase in phosphorylation at S1448, presumably also by ERK. Previous studies have shown that PKA phosphorylation close to a KIM site might inhibit ERK binding (Houslay and Kolch, 2000), although at present we do not know whether PKA phosphorylation can similarly affect the ability of hScrib to interact with ERK. ERK1 (p44) and ERK2 (p42)

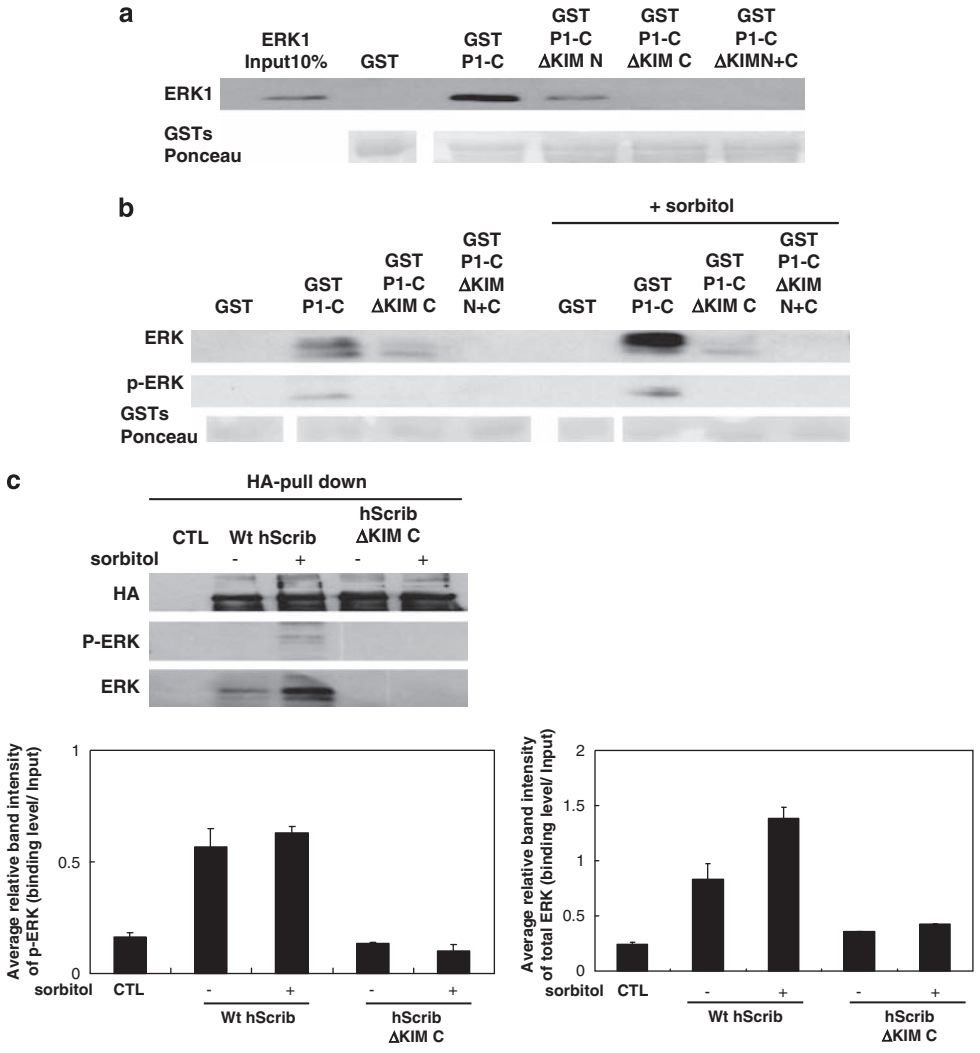

Figure 5 hScrib interacts directly with ERK through the two KIM sites. (**a**) Commercially available purified ERK1 was incubated with the GST-PDZ1-C wild-type and the ΔKIM N, ΔKIM C and the ΔKIM N + C mutants and bound ERK1 ascertained by western blotting. The lower panel shows the Ponceau stain of the nitrocellulose membrane. (**b**) HEK293 cell extracts from untreated and sorbitol-exposed cells were incubated with the GST-PDZ1-C wild-type and the two KIM site mutant GST fusion proteins (single and double) immobilized on glutathione-agarose beads. The bound proteins were analysed by western blotting with the anti-phospho ERK1/2 antibody and the anti-ERK1/2 antibody. The input GSTs are shown in the Ponceau stain of the nitrocellulose membrane. (**c**) HEK293 cells transfected with pcDNA3.1 (CTL), HA-hScrib or the HA-hScribΔKIM C mutant and the cells were then either incubated with or without 0.3 M sorbitol for 10 min, after which the cells were extracted and immunoprecipitated with anti-HA agarose beads. Co-immunoprecipitated proteins were then analysed by western blotting for anti HA-Scrib and anti-pERK/total ERK. The lower histogram shows the quantitative analyses of the intensities of the pERK and total ERK bindings from three independent experiments with s.d. indicated.

have numerous substrates in common, many of which are nuclear and which participate in the transcriptional regulation of a number of different cellular processes (Treisman, 1996). However, ERK1 and ERK2 are not entirely functionally redundant, and our studies confirm this as we found that hScrib S1448 is preferentially phosphorylated by ERK1. At present, we have no information as to what are the functional consequences of ERK or PKA phosphorylation of hScrib. However, we can speculate that this will most likely affect the ability of hScrib to interact with some of its cellular partners, and studies are currently in progress to investigate these aspects further.

Finally, it is worth noting that only in *Homo sapiens* is the organization of the two KIM sites and the corresponding phospho-acceptor sites perfectly well conserved in Scribble. Figure 8 shows the sequence alignment of Scribble from a number of different organisms. It can be seen from this that although the C-terminal KIM and phospho-acceptor site are well conserved among vertebrate species, the N-terminal site is somewhat divergent, whereas in lower organisms neither of the two regulatory elements seem to be conserved. This is particularly true for *Drosophila*, which has been the model organism of choice for many of the studies on hScrib, and suggests a very different

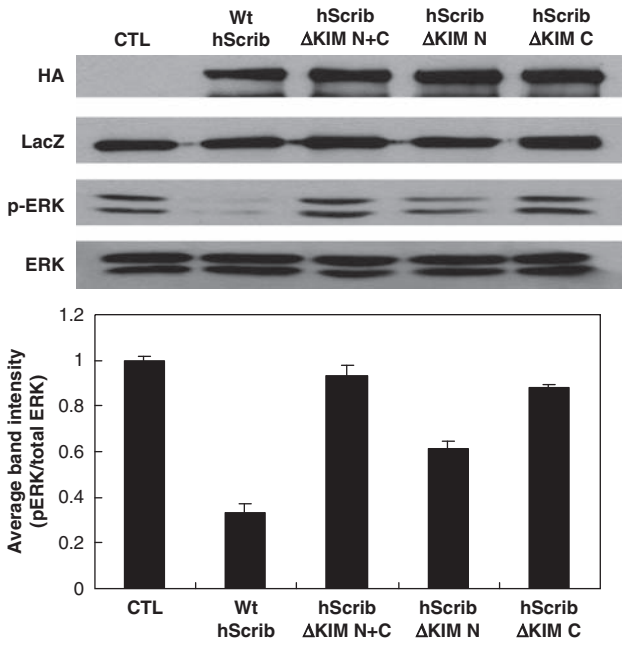

Figure 6 hScrib downregulates ERK activation through a direct interaction. HEK293 cells were transfected with pcDNA3.1 (CTL), HA-tagged wild-type hScrib, ΔKIM N + C, ΔKIM N and ΔKIM C mutants. After 24 h, the cells were harvested and the levels of ERK and phospho-ERK were analysed by western blotting. LacZ was monitored as a control for transfection efficiency. The lower panel shows the quantifications of the pERK/total ERK ratios from at least three independent experiments.

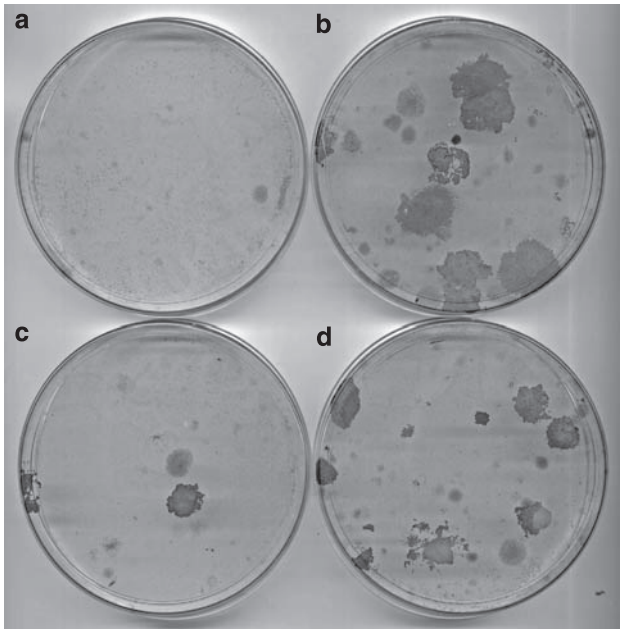

Figure 7 hScrib suppresses human papillomavirus (HPV)-16 E7 and EJ-ras oncogene cooperation in a KIM site-dependent manner. BRK cells were transfected with EJ-ras alone (**a**), HPV-16 E7 plus EJ-ras (**b**), HPV-16 E7 plus EJ-ras and wild-type hScrib (**c**) and HPV-16 E7 plus EJ-ras and the ΔKIM C hScrib mutant (**d**). After 3 weeks, the dishes were fixed and stained and the colonies counted.

Table 1 Suppression of HPV-16 E7 and EJ-ras cooperation by hScrib is KIM site-dependent

	Number of cell colonies		
	Exp 1	*Exp 2*	*Exp 3*
EJ-ras	10	0	0
EJ-ras + 16 E7	52	34	63
EJ-ras + 16 E7 + hScrib	22	11	44
EJ-ras + 16 E7 + hScrib ΔKIMC	30	33	59

Abbreviations: HPV, human papillomavirus; hScrib, human Scribble; KIM, kinase interaction motif; KIM C, KIM C-terminal.
Number of colonies obtained after 3 weeks of cultivation in three independent assays.

form of regulation and function of hScrib between flies and higher organisms. It is also worth noting that only in *Homo sapiens* is the potential regulatory PKA site so closely juxtaposed to the carboxy terminal ERK phospho-acceptor site, and further studies are warranted to determine whether there are any co-regulatory effects in humans of these two kinases on hScrib.

In summary, we have identified a novel regulatory mechanism by which the cell polarity regulator hScrib can directly control the MAPK signalling cascade through a direct protein interaction with ERK. These studies suggest that loss of hScrib expression, which is observed in many tumours, can directly affect continued cell proliferation and cell survival by increasing MAPK activation and nuclear translocation.

Materials and methods

Cells and treatments

HEK293 (human embryonic kidney cells), HaCaT (human keratinocyte) and BRK cells were cultured in Dulbecco's modified Eagles's medium supplemented with 10% fetal bovine serum, penicillin–streptomycin (100 U/ml) and glutamine (300 μg/ml) in a humidified 5% CO_2 incubator. Transfection was carried out using calcium phosphate precipitation as described previously (Graham and van der Eb, 1973) or using Lipofectamine 2000 (Invitrogen, Milan, Italy) according to the manufacturer's protocol. To generate the depleted Scribble cell lines, HaCaT cells were transfected using a pool of short hairpin RNA constructs against hScrib (S2, ScribC) using Lipofectamine 2000 (Invitrogen). The cells were then selected with puromycin (500 ng/ml) and after 4 weeks single colonies were analysed for hScrib expression by immunofluorescence and western blotting, and two such separate colonies (S2, ScribC) were used in this analysis. Parallel transfections and selections were performed using empty vector to generate control clones (TR) that had been subjected to the drug selection. For induction of osmotic shock, the cells were exposed to 0.3 M sorbitol for the times indicated in the text.

Cell transformation assays were performed using BRK cells obtained from 9-day-old Wistar rats with a combination of human papillomavirus-16 E7 and EJ-ras plus the appropriate hScrib expression plasmids. Cells were placed under G418 selection for 3 weeks, and then fixed and stained as described previously (Thomas *et al*., 2005).

Plasmids

The wild-type HA-tagged pcDNA hScrib expression plasmid and the truncated mutant pGEX hScrib PDZ1-C, PDZ1-4

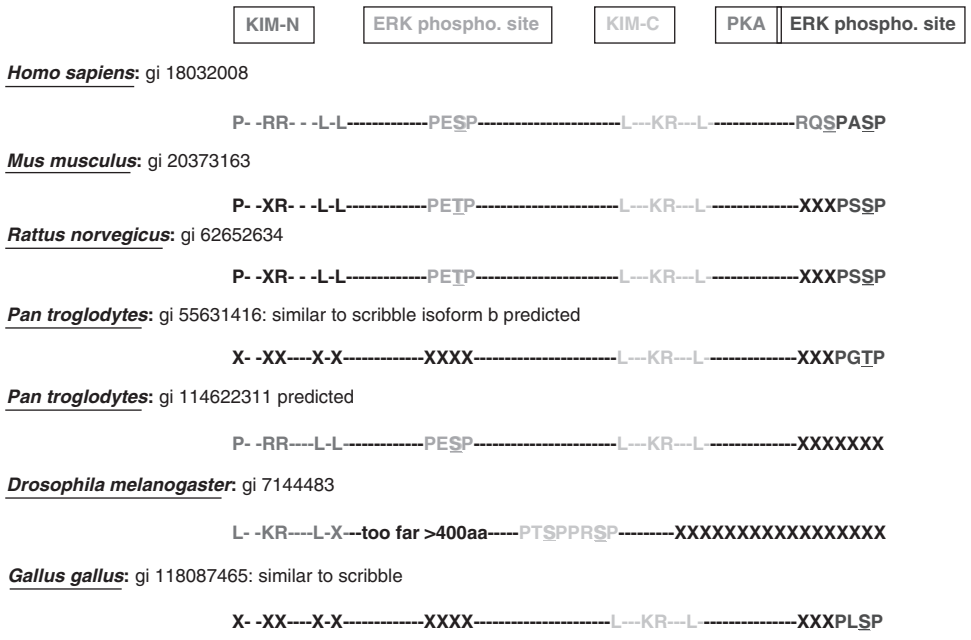

Figure 8 Comparison and sequence alignment of the region of hScrib containing the consensus ERK phosphorylation/binding sites in humans, chimpanzees, mice, rats, chickens and *Drosophila*. There is no evidence for the conservation of the C-terminal hScrib-dependent ERK signalling cascade in non-vertebrate species; however, interestingly, only human Scribble has the two ERK sites.

expression plasmids have been described previously (Thomas *et al*., 2005; Nagasaka *et al*., 2006). The mutations of Ser 853, 1445 and 1448 to either singly, doubly alanine(A) or aspartate(D) or KR, RR to alanine AA mutants in hScrib were performed using the QuikChange XL site-directed mutagenesis kit from Stratagene Cloning Systems (La Jolla, CA, USA) (Celbio, Milan, Italy) according to the manufacturer's instruction. The mutants were confirmed by DNA sequencing.

Antibodies

The following commercial antibodies were used at the dilution indicated: anti-hScrib goat polyclonal antibody (Santa Cruz, Santa Cruz, CA, USA; western blot (WB) 1:1000), anti-p44/42 MAPK (Erk1/2) antibody (Cell Signalling Technology, Danvers, MA, USA; WB 1:1000), anti-phospho p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (Cell Signaling Technology, WB 1:1000), anti-HA monoclonal antibody 12CA5 (Roche, Milan, Italy; WB 1:500), anti-β-galactosidase antibody (Promega, Milan, Italy; WB 1:5000), anti-γ-tubulin monoclonal antibody (Sigma, Milan, Italy; WB 1:5000), anti-p84 mouse monoclonal antibody (Abcam, Cambridge, UK; WB 1:1000), anti-α-tubulin mouse monoclonal antibody (Abcam, WB 1:1000) and anti-E-Cadherin rabbit polyclonal antibody (Santa Cruz, WB 1:500).

Immunofluorescence and microscopy

For immunofluorescence, cells were grown on glass coverslips and fixed in 3.7% paraformaldehyde in phosphate-buffered saline (PBS) for 20 min at room temperature. After washing in PBS, the cells were permeabilized in PBS/0.1% Triton for 5 min, washed extensively in PBS and then incubated with primary antibody diluted in PBS for 1 h followed by the appropriately conjugated secondary antibodies. Secondary antibodies conjugated to Alexa Fluor 488 or 548 were obtained from Invitrogen. The cells were then washed several times in water and mounted on glass slides. Cells were visualized using a Zeiss Axiovert 100 M microscope (Zeiss, Milan, Italy) attached to a LSM 510 confocal unit.

Small interfering RNA transfection

HEK293 cells were seeded on 6 cm dishes and transfected using Lipofectamine 2000 (Invitrogen) with control small interfering RNA against Luciferase (siLuc), or small interfering RNA against hScrib sequences (Dharmacon, Lafayette, CO, USA). At 48 h after transfection, cells were harvested and total cells extracts or cell fractionated extracts were then analysed by western blotting.

In vitro *kinase assays*

Purified GST fusion proteins were incubated with commercially purified ERK1, ERK2, JNK1 (Cell Signaling Technology) or PKA (Promega) for 20 min at 30 °C in phosphorylation buffer (0.25 M Tris pH 7.5, 1 M $MgCl_2$, 3 M NaCl, 0.3 mM aprotinin and 1 mM Pepstatin) supplemented with 56 nM [^{32}P] γ-ATP (Perkin Elmer, Waltham, MA, USA) and 10 mM ATP following the manufacturer's instruction. After extensive washing, the phosphorylated proteins were monitored by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and autoradiography.

Phospho-mapping analyses

HEK293 cells were transfected with HA-tagged Scrib and after 24 h left untreated or exposed to sorbitol for 30 min. After this time, the cells were extracted and proteins immunoprecipitated with anti-HA agarose beads, separated on SDS–PAGE and the silver-stained gel slice corresponding to hScrib was excised. Phospho-mapping mass spectroscopy was then performed using NextGen Sciences (Ann Arbor, MI, USA).

Subcellular fractionation assays

Differential extraction of HEK293 cells to obtain cytoplasmic, nuclear and membrane fractions was performed using the

Calbiochem ProteoExtract Fractionation Kit (Calbiochem, Milan, Italy) according to the manufacturer's instructions. To inhibit phosphatase activity during the preparation of cell lysates, phosphatase inhibitors (1 mM Na_3VO_4, 1 mM β-glycerophosphate, 2.5 mM sodium pyrophosphate and 1 mM sodium fluoride) were also included.

Immunoprecipitation and western blotting

Total cellular extracts were prepared by directly lysing cells from dishes in SDS lysis buffer. Alternatively, cells were lysed in either E1A buffer (25 mM HEPES pH 7.0, 0.1% NP-40, 150 mM NaCl, plus protease inhibitor cocktail; Calbiochem) or RIPA buffer (50 mM Tris–HCl pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA, plus protease inhibitor cocktail; Calbiochem). The supernatant (soluble fraction), pellet (insoluble fraction) and the whole cells extracts were analysed by SDS–PAGE and western blotting. For immunoprecipitations, total cell lysates were transferred into a tube of equilibrated EZview Red Anti-HA Affinity Gel beads (Sigma), and incubated for 2 h at 4 °C. Immunoprecipitates were extensively washed four times in lysis buffer and solubilized in SDS–PAGE sample buffer. For western blotting, 0.45 μm nitrocellulose membrane (Schleicher and Schuell, Milan, Italy) was used and membranes were blocked for 1 h at 37 °C in 10% milk/PBS followed by incubation with the appropriate primary antibody diluted in 10% milk/0.5% Tween 20 for 1 h. After several washings with PBS 0.5% Tween 20, secondary antibodies conjugated with horseradish peroxidase (DAKO, Milan, Italy) in 10% milk/0.5% Tween 20 were incubated for 1 h. Blots were developed using Amersham enhanced chemiluminescence reagents (Amersham, Milan, Italy) according to the manufacturer's instructions.

Conflict of interest

The authors declare no conflict of interest.

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