Small GTPases 1:2, 108-112; September/October 2010; © 2010 Landes Bioscience

The mechanisms and implications of hScrib regulation of ERK

Kazunori Nagasaka,[1,2,*] Paola Massimi,[2] David Pim,[2] Vanitha Krishna Subbaiah,[2] Christian Kranjec,[2] Shunsuke Nakagawa,[1] Tetsu Yano,[1] Yuji Taketani[1] and Lawrence Banks[2,*]

[1]Department of Obstetrics and Gynecology; Graduate School of Medicine; University of Tokyo; Tokyo, Japan; and [2]International Centre for Genetic Engineering and Biotechnology; Trieste, Italy

Scribble is a potential tumor suppressor protein, whose loss is a frequent event in late stage cancer development. In both Drosophila and mammalian model systems, Scribble has been shown capable of regulating cell polarity, cell proliferation and apoptosis. Although several interacting partners, including βPiX, have been identified that help to explain how Scribble can regulate cell polarity and migration, little is known about how Scribble can control cell proliferation. Recent work from our laboratory has shown that Scribble can directly regulate the ERK signaling pathway. This is mediated by a direct protein-protein interaction between Scribble and ERK, which has two components. In the first, Scribble appears to anchor ERK at membrane-bound sites, with the loss of Scribble enhancing ERK nuclear translocation. In the second, Scribble can decrease the levels of active phosphorylated ERK, a function that is dependent upon the ability of Scribble to bind ERK directly. One of the consequences of this activity of Scribble is the inhibition of EJ-ras induced cell transformation. These results provide some of the first direct mechanistic information on how Scribble can regulate cell proliferation and, furthermore, they provide indications as to the identity of other signaling intermediates that may be recruited by Scribble to directly regulate mitogenic signaling pathways.

Key words: cell proliferation, scribble, ERK, phosphorylation

Submitted: 09/02/10

Revised: 09/12/10

Accepted: 09/15/10

Previously published online: www.landesbioscience.com/journals/smallgtpases/article/13649

DOI: 10.4161/sgtp.1.2.13649

*Correspondence to: Lawrence Banks andKazunori Nagasaka;
Email: banks@icgeb.org
and nagasakak-tky@umin.ac.jp

Extra View to: Nagasaka K, Pim D, Massimi P, Thomas M, Tomaic V, Subbaiah VK, et al. The cell polarity regulator hScrib controls ERK activation through a KIM site-dependent interaction. Oncogene 2010; 29:5311–21; PMID: 20622900; DOI: 10.1038/onc.2010.265.

The first evidence linking cell polarity and cell proliferation control came from studies in Drosophila and *Caenorhabditis elegans*, and provided some of the first indications of the potential functional relevance of this link in the development of human tumors. These studies identified three multi-protein complexes as central to the control of this pathway: the Par complex, the Scribble complex and the Crumbs complex.[1-4] In Drosophila these proteins cooperate in the regulation of polarity and proliferation.[1,5,6] In humans, the functions of these proteins are less clear, although several studies have reported that the loss or overexpression of certain components of the network is linked to cancer development.[7,8] As we will see in the following discussion, the molecular basis for some of these observations, are beginning to be unraveled. Studies with human tumor viruses have provided further compelling evidence of potential tumor suppressor functions for the cell polarity regulatory module. For example, the high-risk Human Papillomaviruses (HPVs), which are the causative agents of cervical cancer, specifically target the Scribble complex;[9] with the HPV-16 E6 oncoprotein degrading Scribble, whereas the HPV-18 E6 oncoprotein degrades Discs Large (Dlg), another component of the complex.[10] Perhaps most importantly, HPV-16 is the cause of over 60% of all cervical cancers,[11] and this particular link to Scribble is therefore intriguing. Interestingly, in the evolutionarily-related Rhesus Papillomavirus (RhPV), which causes cervical cancer in the Rhesus macaque, this complex is also targeted, but in this case Par3 is the viral substrate.[12] These studies suggest that the pathways of polarity and proliferation control that are regulated by the Scribble and Par complexes are functionally relevant for Papillomavirus-induced cervical cancers.

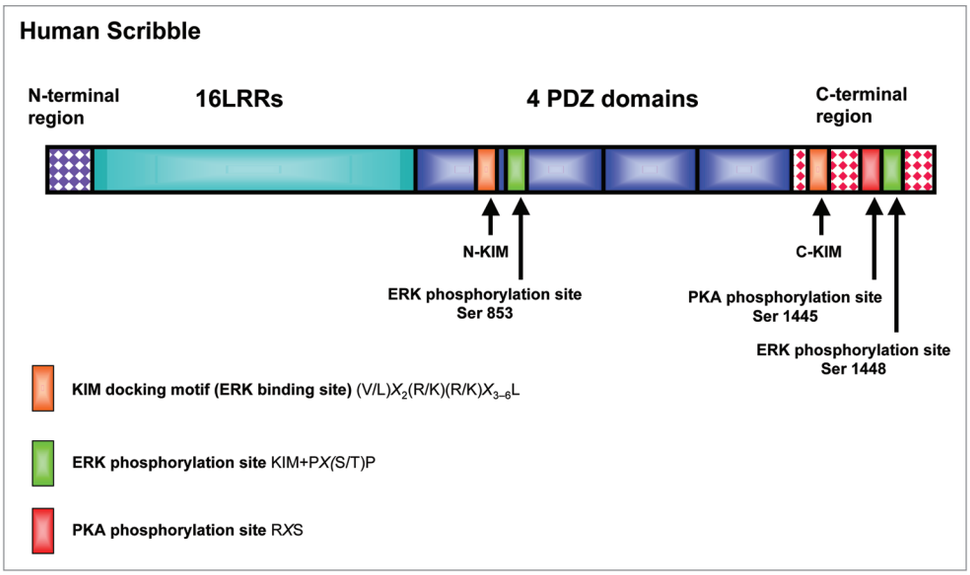

Figure 1. Schematic diagram showing the human Scribble protein. The major functional domains on Scribble are shown including the Leucine Rich Repeats (LRRS) and the 4 PDZ domains. Also shown are the N and C terminal KIM sites and their corresponding phospho-acceptor sites (S853 and S1448), together with the location of the PKA phospho-acceptor site (S1445).

The Scribble protein is depicted schematically in **Figure 1**, and it comprises 1,630 amino acids. It is classified as a LAP family member, and contains a series of 16 leucine rich repeats (LRRs) at the N terminus, which are required for correct localization of Scribble to the basolateral membrane, and these are followed by 4 PDZ (PSD95/Dlg/ZO-1) domains that function as sites of protein-protein interactions.[13,14] Indeed, Scribble is localized primarily at sites of cell-cell contact, where it is proposed to act as a molecular scaffold. Loss of Scribble expression has been reported in colon, breast and uterine tumors,[15-17] and the human equivalent can functionally replace the Drosophila homologue, resulting in a restoration of the normal polarity and proliferation controls that are lost in Scribble-null flies.[18] One of the major functions of Scribble in human cells is the regulation of cell polarity and motility, which seems to be achieved, in part, through its association with the βPiX.[19] βPix is a Guanine nucleotide exchange factor (GEF) for the Rac/Cdc42 small GTPase complex,[20] and thereby directly regulates actin remodeling in response to various stimulatory cues.[21-23] Through this interaction, Scribble controls Cdc42 localisation during cell migration.[24] Scribble also has the capacity to regulate apoptotic signaling in mammary epithelial cells and in Drosophila eye imaginal discs, where, depending on the particular experimental setting, it can exhibit both pro-and anti-apoptotic potential.[25,26]

We were intrigued by previous studies that had linked Scribble to the control of mitogenic signaling pathways; with loss of Scribble clearly contributing to oncogenic Ras-induced cell transformation, both in Drosophila and in mammalian cells.[26-28] Analysis of the Scribble sequence revealed the existence of two perfect kinase interaction motifs (KIM sites; see **Fig. 1**), suggesting that Scribble had the propensity to interact with extracellular signal-related kinase (ERK). In our study, we showed that Scribble is a strong substrate for phosphorylation by ERK and also by protein kinase A (PKA), consistent with recent studies showing that Scribble is heavily phosphorylated at these sites, and also elsewhere in the molecule.[29] Most importantly, however, we found that Scribble forms a strong direct interaction with ERK that is absolutely dependent upon the integrity of the two identified KIM sites, although the interaction with the carboxy terminal site is much stronger. In a number of different experimental settings, we verified that Scribble could directly regulate ERK activation, as determined by the level of active phosphorylated ERK expressed in cells in the presence or absence of Scribble; and we demonstrated the importance of the KIM sites for this regulation. Thus, under circumstances where Scribble can no longer interact with ERK, we observed an increase both in the levels of ERK nuclear translocation, and in the levels of phosphorylated active ERK. This activity of Scribble also correlated with the capacity of Scribble to inhibit oncogenic Ras-induced cell transformation in an oncogene cooperation assay.

These studies now raise a number of interesting issues. Obviously, the prevalence of potential regulatory phospho-acceptor sites on Scribble is worthy of further investigation. It was intriguing that we found an apparent link between the two very closely located ERK and PKA phospho-sites at S1445 and S1448, respectively. Under no circumstances were we able to observe dual phosphorylation of both sites in vivo, suggesting that one is mutually exclusive of the other. This suggests an important regulatory function, and our current studies are using proteomic approaches to analyse changes in the capacity of the different phosphorylated forms of Scribble to interact with different cellular targets. However, phosphorylation seems to have an effect on the pattern of Scribble expression within cells,

Table 1. Sequence alignments of the scribble protein from different species

	KIM-N	ERK phospho. N site	KIM-C	PKA phospho. site	ERK phospho. C site
Homo sapiens gi18032008	P- -RR- - -L-L	PESP	L---KR---L-	RQS	PASP
Mus musculus gi20373163	P- -XR- - -L-L-	PETP	L---KR---L-	XXX	PSSP
Rattus norvegicus gi 62652634	P- -XR- - -L-L-	PETP	L---KR---L-	XXX	PSSP
Gallus gallus gi 118087465	X- -XX----X-X-	XXXX	L---KR---L-	XXX	PLSP
Drosophila melanogaster gi 7144483	L- -KR----L-X-	PTSPPRSP too far >400aa-	X- -XX----X-	XXX	XXXX

Shown are the consensus sequences for the N- and C-terminal KIM sites, the corresponding phospho-acceptor sites and the associated PKA phospho-acceptor site.

suggesting that differential phosphorylation at these sites may affect Scribble's localization, and hence its function. In addition, it has been shown that the activation of Cdc42, which controls many aspects of cell polarization at the leading edge,[21,30,31] in turn requires the Scribble PDZ domains (implying βPiX involvement) and the Scribble carboxy-terminal region.[24] This suggests that the association of Scribble with ERK through the carboxy terminal KIM site may contribute both to the regulation of directional cell migration, and to the maintenance of cellular polarity. Finally, these studies also raise important questions from an HPV E6 point-of-view. We had previously shown that Dlg was rendered more susceptible to HPV E6 targeting as a result of certain phospho-modifications.[32] Studies are now ongoing to determine whether similar modifications of Scribble might also affect its susceptibility to HPV oncoprotein targeting, which might have important implications for the capacity of the virus to induce malignancy.

Since potential regulation of the ERK signaling cascade by Scribble has been reported in Drosophila and in mammalian cells, it is worth considering the degree of evolutionary conservation in the Scribble KIM sites and the corresponding phospho-acceptor sites. As can be seen from **Table 1**, the carboxy terminal KIM site is conserved through mammals to birds. However, this site is absent in Drosophila, as is the corresponding ERK phospho-acceptor site. Conversely, the N terminal KIM site is conserved in mammals, but is absent in birds, and there is only a weak consensus KIM site in Drosophila. The loss of Scribble is known to cooperate with an activated ERK signaling pathway to induce cell transformation in Drosophila. However the differences between flies and mammals suggest that this may not occur through the same mechanisms as are seen in mammalian cells. In Drosophila the loss of Scribble affecting the levels of JNK activation may be the more likely cause of these phenotypes.[26,33] Finally, it is also worth noting that the PKA phospho-acceptor site is unique to human Scribble, and this suggests a further fine-tuning of Scribble function in human cells, which does not occur in other organisms. How relevant this may be to potential tumor suppressor mechanisms remains to be determined, but this is an interesting possibility.

Our studies have demonstrated that Scribble can inhibit the activation of ERK. We proposed two possible mechanisms by which Scribble might achieve this: either by inhibition of ERK phosphorylation by upstream kinases, or by recruitment of a protein phosphatase that can directly inhibit ERK phosphorylation. We favor the latter hypothesis, as we consistently observe that Scribble can reduce ERK phospho-levels below base-line, suggesting an active de-phosphorylation of ERK, as opposed to inhibition of ERK phosphorylation. Studies are currently under way to identify potential phosphatase-bound partners of Scribble, and our preliminary proteomic analyses have identified several such candidates. Thus, one could propose that Scribble can interact with ERK at defined sites within the cell, and at the same time recruit a protein phosphatase which, in turn, can regulate levels of ERK activation; a schematic model of this is shown in **Figure 2**. Should this prove to be the mechanism by which Scribble can control ERK activation, this also has potentially important implications for some of Scribble's other interacting partners, a number of which are listed in **Table 2**. Perhaps one of the most important of these is βPix, which is itself intimately linked to the Ras/ERK signaling pathway.[34] Therefore Scribble could conceivably act as a platform for phosphatase recruitment to different signaling components, and thus exert modulatory activities in a direct fashion, rather than acting in the rather static scaffolding manner that has often been proposed. Further studies will be aimed at further defining the role of Scribble in these signaling complexes.

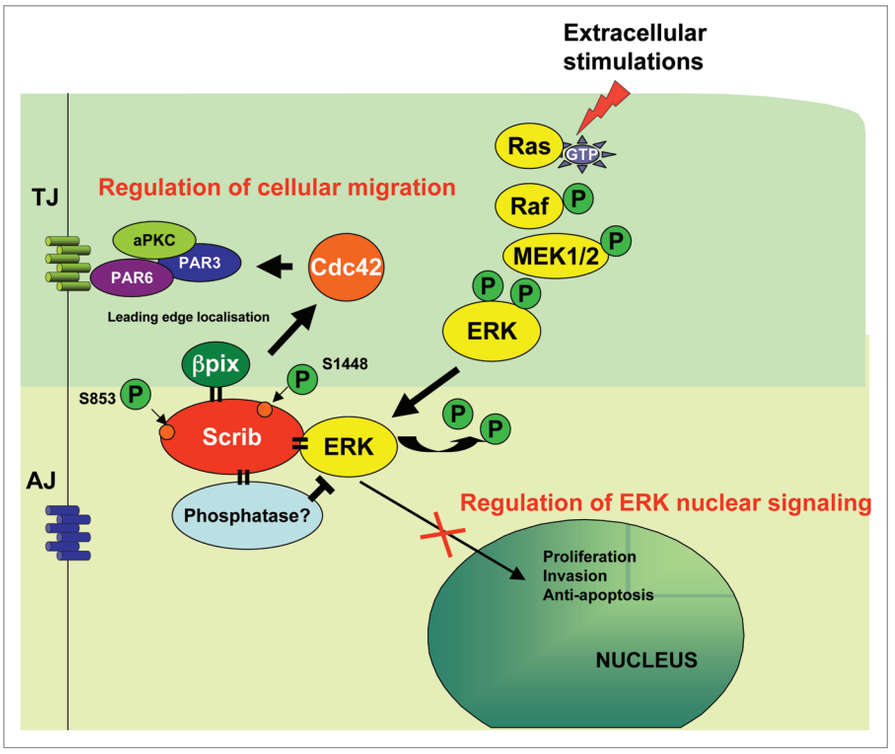

Figure 2. Scribble integrates signaling pathways to control polarized cell migration, cell proliferation and cell invasion. Scribble interacts directly with ERK and βPix. It controls directional migration through regulating the activity of Cdc42 via βPix and also controls the ERK signaling cascade through controlling ERK activity and localisation. This activity of Scribble has implications for controlling cell proliferation and invasion, and we propose that Scribble regulation of ERK is achieved by the direct recruitment of a phosphatase by Scribble. The consequences of ERK phosphorylation of Scribble at S853 and S1448 are currently unknown.

Table 2. Known interacting partners of scribble and their sites of interaction on the scribble protein

	Binding site	References
βpix	4PDZ domains	Audebert et al., 2004
ERK	2 Kinase interaction motifs (KIM)	Nagasaka et al, 2010
HPV16E6	PDZ3	Nakagawa and Huibregtse., 2000 Thomas et al., 2005
βcatenin	4PDZ domains	Sun et al., 2009
APC (adenomatous polyposis coli)	PDZ1, PDZ4	Takizawa et al., 2006 Nagasaka et al., 2006
TRIP6	PDZ3	Petit et al., 2005
ZO-2	PDZ3, PDZ4	Metais et al., 2005
Keratin 18	PDZ2< PDZ1< PDZ3	Phua et al., 2009
Vimentin	PDZ2< PDZ1< PDZ3	Phua et al., 2009
TBEV NS5	PDZ4	Werme et al., 2008
Influenza virus NS1	PDZ1+PDZ2	Liu et al., 2010
Lgl-2	LRR domains	Kallay et al., 2006

Scribble directly interacts with βPix,[20] ERK,[35] high-risk HPV E6,[10,36] βcatenin,[37] Adenomatous Polyposis Coli,[38,39] TRIP6,[40] ZO-2,[41] Keratin 18,[42] Vimentin,[42] Tick-Borne Encephalitis Virus NS5,[43] avian Influenza virus NS1,[44] and Lgl2.[45]

Acknowledgements

Work in the LB laboratory is supported by research grants from the Associazione Italiana per la Ricerca sul Cancro, Association for International Cancer Research and Telethon. K.N. was supported by the Yoshida (YKK) Scholarships Foundation. We are grateful to Miranda Thomas for comments on the manuscript.

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