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Mst4 and Ezrin Induce Brush Borders Downstream of the Lkb1/Strad/Mo25 Polarization Complex

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SUMMARY

The human Lkb1 kinase, encoded by the ortholog of the invertebrate Par4 polarity gene, is mutated in Peutz-Jeghers cancer syndrome. Lkb1 activity requires complex formation with the pseudokinase Strad and the adaptor protein Mo25. The complex can induce complete polarization in a single isolated intestinal epithelial cell. We describe an interaction between Mo25α and a human serine/threonine kinase termed Mst4. A homologous interaction occurs in the yeast *Schizosaccharomyces pombe* in the control of polar tip growth. Human Mst4 translocates from the Golgi to the subapical membrane compartment upon activation of Lkb1. Inhibition of Mst4 activity inhibits Lkb1-induced brush border formation, whereas other aspects of polarity such as the formation of lateral junctions remain unaffected. As an essential event in brush border formation, Mst4 phosphorylates the regulatory T567 residue of Ezrin. These data define a brush border induction pathway downstream of the Lkb1/Strad/Mo25 polarization complex, yet separate from other polarity events.

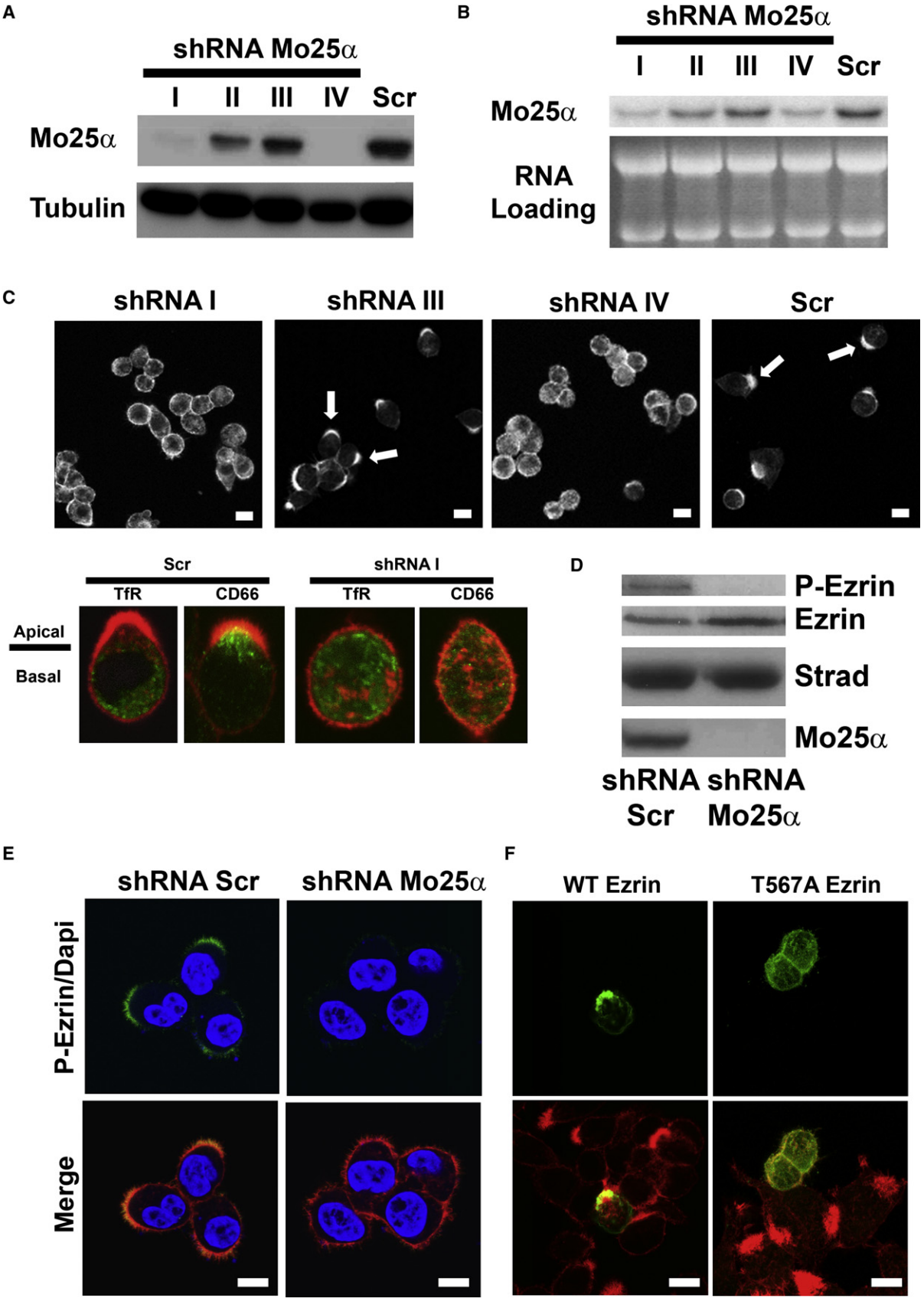
INTRODUCTION

The *lkb1* tumor suppressor gene encodes a serine/threonine kinase, mutated in around 90% of Peutz-Jeghers patients (Giardiello et al., 2000). Studies in *Caenorhabditis elegans* (Watts et al., 2000; Kemphues et al., 1988) and *Drosophila melanogaster* (Martin and St Johnston, 2003) have identified the Lkb1 ortholog *par4* as a master regulator of polarity in multiple cellular processes. We have shown previously that Lkb1, itself a very weak, nuclear kinase, forms a complex with the pseudokinase Stradα or β and the putative adaptor protein Mo25α or β (Baas et al., 2004; Boudeau et al., 2003). In this cytoplasmic complex, Lkb1 is stable and highly active.

Members of a small family of kinases which include the Par1/Mark and AMPK kinases can be activated by Lkb1 by direct phosphorylation of their activation loop (Lizcano et al., 2004). Through phosphorylation of AMPK, the Lkb1 complex controls cellular metabolism by enhancing glucose uptake, glycolysis, and fatty acid oxidation or by inhibiting energy-consuming anabolic processes (Alessi et al., 2006). Independently, we have shown that human Lkb1 functionally resembles the invertebrate polarity gene Par4, in that it plays a central role as an inducer of polarization in epithelial cells (Baas et al., 2004). The effect of Lkb1 on polarization is not restricted to cells of epithelial origin; it has now, for instance, been shown to be relevant for neuronal cells in the mouse cerebral cortex (Barnes et al., 2007; Shelly et al., 2007). The effects on polarity are—at least in part—mediated by phosphorylation of the microtubule affinity-regulating kinases Mark1–4 in vertebrates and Par1 in the model organisms *D. melanogaster* and *C. elegans*. Par1/Mark kinases in turn regulate cellular polarization by modulating the microtubule cytoskeleton (Drewes et al., 1997).

The adaptor protein Mo25 is conserved from yeast to human. In the fission yeast *Schizosaccharomyces pombe*, Mo25 and the Nak1 kinase play an essential role in polarized growth and accumulation of F-actin at the cell tip during S and G2 phases (Mendoza et al., 2005; Kanai et al., 2005). Nak1 has five human homologs: Mst1–4 and Ysk1. Mst1 and Mst2 may play a role in apoptosis (Graves et al., 1998; Lee et al., 2001). Mst3 and Ysk1 have an apparent role in migration, a process that may be interpreted as a form of cellular polarization (Lu et al., 2006; Preisinger et al., 2004). The function or downstream targets of Mst4 remain unknown, although it has been shown that this kinase interacts with the Golgi matrix protein GM130 (Preisinger et al., 2004) and resides on Golgi vesicles in the cell.

One of the polarity-related phenomena that is induced by Lkb1 in intestinal epithelial cells is the induction of brush borders at the apical domain (Baas et al., 2004). Independent studies have indicated a central role for Ezrin in the formation of brush borders in mouse retinal and intestinal cells and in luminal epithelia of *C. elegans* (Bonilha et al., 2006; Gobel et al., 2004; Saotome et al., 2004). Ezrin is a member of the ERM (Ezrin, Radixin, Moesin) family. Ezrin can exist in an "open"/active or "closed"/inactive state (Niggli and Rossy, 2008). In the open conformation, Ezrin can interact with the plasma membrane, F-actin, and multiple signaling molecules (Niggli and Rossy, 2008), creating a platform for the actin-rich brush borders which are particularly prominent on intestinal epithelial cells (Saotome et al., 2004). Activation of Ezrin is mediated by initial PIP2 binding and


A
shRNA Mo25α
I II III IV Scr
Mo25α
Tubulin
B
shRNA Mo25α
I II III IV Scr
Mo25α
RNA Loading
C
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shRNA III
shRNA IV
Scr
Scr
shRNA I
TfR CD66 TfR CD66
Apical
Basal
D
P-Ezrin
Ezrin
Strad
Mo25α
shRNA Scr
shRNA Mo25α
E
shRNA Scr
shRNA Mo25α
P-Ezrin/Dapi
Merge
F
WT Ezrin
T567A Ezrin

subsequent phosphorylation of threonine 567 (Fievet et al., 2004). Mutations of this residue to either alanine or aspartic acid has been shown to result in an inactive or an active Ezrin protein, respectively (Fievet et al., 2004).

Here we show that Mo25 interacts with Mst4 and that formation of an active Lkb1/Strad/Mo25 complex results in the translocation of Mst4 from Golgi vesicles to the subapical domain of polarizing cells. Translocation of Mst4 induces the phosphorylation of the actin remodeler Ezrin at its regulatory residue T567. Based on this study, we propose a pathway in which Mst4 relays a polarizing signal from the Lkb1 complex to Ezrin, resulting in the formation of brush borders.

RESULTS

Mo25 Is Required for Lkb1-Strad-Induced Brush Border Formation

Previously, we reported that the induced expression of Strad in intestinal epithelial cells leads to stabilization of Lkb1 in a complex with Strad and Mo25 and in its subsequent activation. We utilized Ls174T intestinal epithelial cells, engineered to stably express a Myc-tagged Lkb1 and a doxycycline-inducible, Flag-tagged Stradα (Baas et al., 2004). Induction of Stradα in these so-called W4 cells leads to rapid polarization of isolated individual cells, as evidenced by the formation of an apical brush border as well as by the correct sorting of apical and basolateral markers and of components of junctional complexes (Baas et al., 2004).

Mo25 is a component of the active Lkb1 complex (Boudeau et al., 2003), yet the involvement of Mo25 for Lkb1-induced polarization in living cells has not been addressed previously. Two Mo25 genes exist in the mammalian genome, Mo25α and β. Of these, only Mo25α was found to be expressed in Ls174T cells (data not shown). To study the involvement of Mo25α in induction of polarity, we downregulated Mo25α expression by means of retrovirally mediated expression of shRNA in W4 cells. Upon induction of Stradα by doxycycline, W4 cells polarized and formed brush borders, as visualized with CD66 and TfR and the F-actin-binding compound phalloidin-TRITC, respectively. W4 cells with reduced expression of Mo25α (Figures 1A and 1B) lost their ability to polarize and form brush borders in response to Lkb1 activation (Figure 1C), whereas W4 cells expressing scrambled shRNA polarized properly. This demonstrated that Mo25α is essential for Lkb1-induced polarization and brush border formation.

Phosphorylated Ezrin Mediates Lkb1-Induced Brush Border Formation

To study the involvement of Ezrin phosphorylation in the formation of brush borders as induced by activated Lkb1, we performed western blot and confocal analysis of polarized W4 cells stained for phosphorylated Ezrin. By western blot analysis, we found a reduced signal for phosphorylated Ezrin in shMO25 cells compared to control cells (Figure 1D). Furthermore, a clear signal for phosphorylated Ezrin-T567 occurred specifically in the brush borders of these cells and not in the Mo25α knockdown cells (Figure 1E). This suggested that phosphorylation of Ezrin is a key downstream event in Lkb1/Strad/Mo25-induced brush border formation.

We then exogenously expressed wild-type (WT) or the dominant-negative Ezrin mutant T567A (Fievet et al., 2004) in W4 cells and induced the expression of Stradα for 24 hr. Brush border formation was visualized by actin staining, whereas Ezrin was detected with an anti-VSV antibody. Cells expressing WT Ezrin retained their ability to form brush borders (45/50), whereas brush borders were completely absent in T567A Ezrin-expressing cells (0/50) (Figure 1F). This implied an involvement of phosphorylated Ezrin in Lkb1-induced brush border formation.

Identification of Mst4 as a Potential Mediator between Lkb1 and Ezrin

The sequence around T567 of Ezrin does not resemble an optimal Lkb1 phosphorylation site (Boudeau et al., 2003), suggesting that Ezrin is not a direct downstream target of Lkb1. Indeed, we were unable to demonstrate direct phosphorylation of Ezrin by the Lkb1 complex (data not shown). Therefore, we pursued the identification of intermediary proteins by two different approaches: (1) by performing a yeast two-hybrid screen with Mo25α as bait, and (2) by searching for kinases capable of phosphorylating Ezrin at T567 in vitro.

Mo25 structurally resembles armadillo/heat repeat adaptor proteins such as β-catenin and importins (Milburn et al., 2004). Its C-terminal hydrophobic pocket binds a Trp-Glu-Phe sequence at the C terminus of Strad (Milburn et al., 2004). We hypothesized that the remainder of the Mo25 molecule could serve as a docking platform for effector molecules of the Lkb1 complex. To identify such interactors, we performed a yeast two-hybrid screen with full-length Mo25α on a human placenta library. The screen was validated by the observation that the most frequently identified

Figure 1. Mo25α and Ezrin Mediate Lkb1-Induced Polarization of W4 Cells

(A) Protein expression of Mo25α in W4 cells expressing shRNA for Mo25α. W4-derived lysates expressing pRetrosuper with shRNA against Mo25α (sequences I–IV) or a scrambled sequence (Scr) were blotted and immunostained for Mo25α and tubulin.

(B) mRNA expression of Mo25α in W4 cells expressing shRNA for Mo25α. W4-derived RNA expressing pRetrosuper with shRNA against Mo25α (sequences I–IV) or a scrambled sequence (Scr) were blotted and probed for Mo25α.

(C) Reduced expression of Mo25α in W4 cells results in loss of polarization. W4 cells with shRNA against Mo25α or a scrambled sequence (Scr) were treated with doxycycline for 24 hr, fixed, and immunostained for apical CD66 (green), basal lateral CD71/TfR (green), and actin (red). Arrows indicate brush borders. The scale bars represent 10 μm.

(D) Activation of the Lkb1-Mo25α pathway induces the phosphorylation of Ezrin. W4 and shRNA Mo25α cells were treated with doxycycline for 24 hr and subsequently subjected to western blot analysis. Lysates were stained for phosphorylated Ezrin (P-Ezrin), Ezrin, Strad, and Mo25α.

(E) Activation of the Lkb1-Mo25α pathway induces the phosphorylation of Ezrin in brush borders. W4 and shRNA Mo25α cells were treated with doxycycline for 24 hr and subsequently fixed and stained for phosphorylated Ezrin (P-Ezrin; green), actin (red), and DNA (blue). The scale bars represent 5 μm.

(F) Ezrin phosphorylation is essential for brush border formation. W4 cells expressing wild-type (WT) Ezrin or the non-phospho mutant Ezrin T567A were treated with doxycycline for 24 hr, fixed, and immunostained for actin (red) and VSV-tagged Ezrin (green). The scale bars represent 5 μm.

A

Gene	Number of clones
Dlg5	21
Strad	18
Mst4	16
RANBP5	13
Exophillin	7
SNRP70	5
ARHGAP18	5
PKCBP1	3
Citron	3
RalGDS	1
STK24/Mst3	1

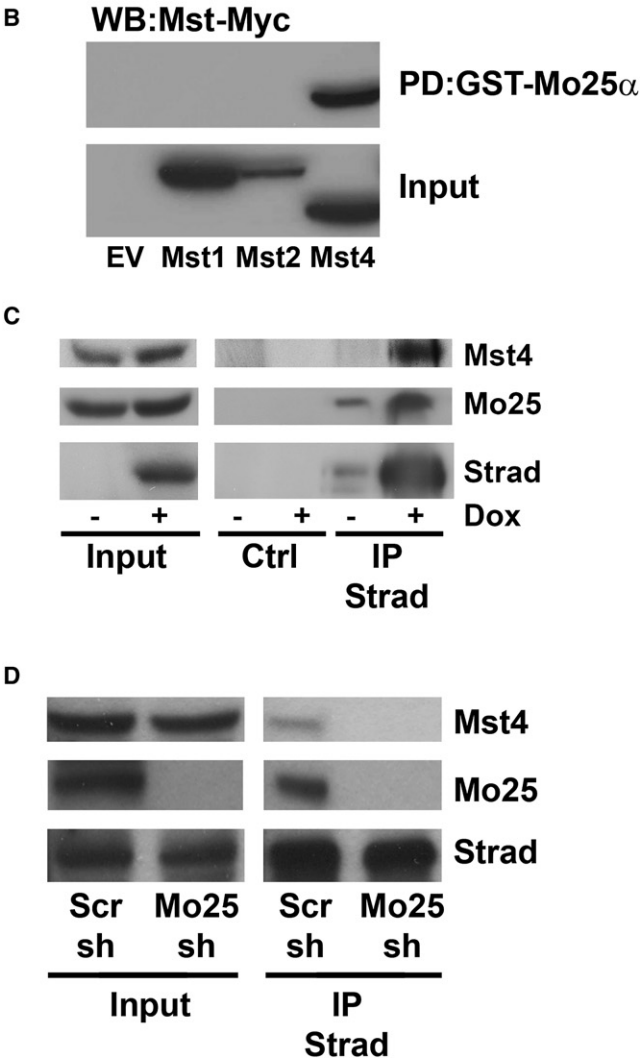


Figure 2. Mst4 Interacts with Mo25α

(A) List of putative Mo25α-interacting genes as identified by yeast two-hybrid screen.

(B) GST-Mo25α interacts specifically with Mst4. GST-Mo25α pull-down assays (PD) were performed in cell lysates expressing pCDNA-Myc (EV) or Myc-tagged Mst1, Mst2, or Mst4. Mst kinases were detected with anti-Myc in pull-downs and total cell lysates (10% of input) after western blotting (WB).

(C) Endogenous complex formation of Mo25α and Mst4. Immunoprecipitations with control mouse immunoglobulins (Ctrl) or anti-Flag Strad were performed in W4-derived lysates treated with doxycycline (Dox) for 24 hr. Samples were blotted and immunostained for endogenous Mst4, Mo25α, and Flag-tagged Strad.

(D) Endogenous complex formation of Mo25α and Mst4. Immunoprecipitations with anti-Flag Strad were performed in W4 and shRNA Mo25α-derived lysates treated with doxycycline for 24 hr. Samples were blotted and immunostained for endogenous Mst4, Mo25α, and Flag-tagged Strad.

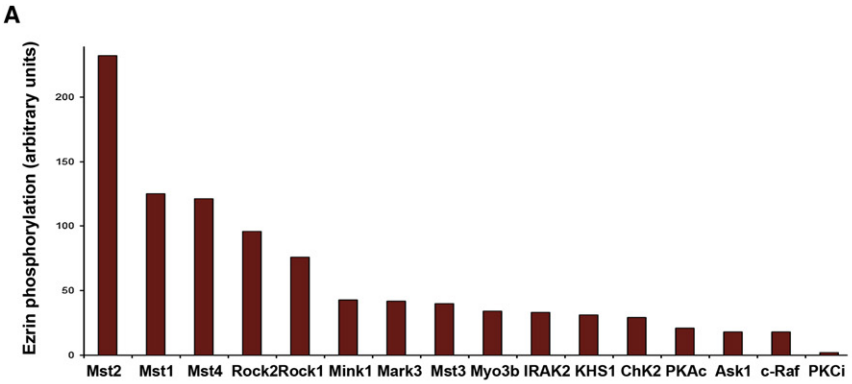
interactor of Mo25α was Stradα (Figure 2A). The kinase Mst4 was another prominent hit in the screen (Figure 2A).

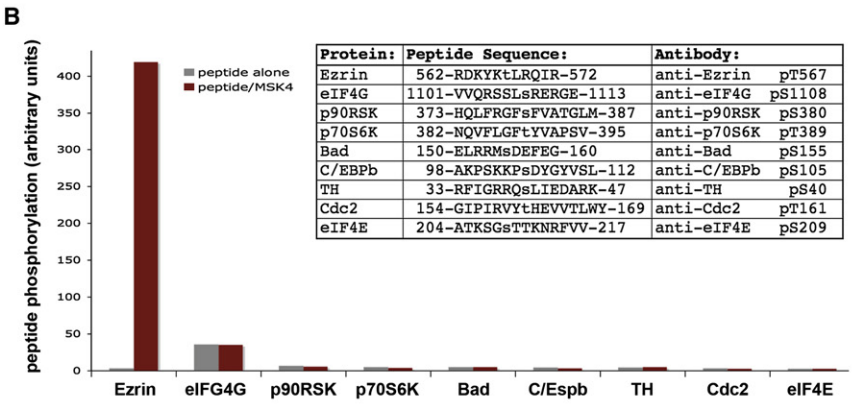
To exclude false positives from the Y2H screen, we exogenously expressed the genes listed in Figure 2A in HEK293T cells and performed pull-down assays with GST-Mo25α in cell lysates. The results of these pull-downs confirmed exclusive binding of GST-Mo25α to Mst4 (Figure 2B) and Stradα (data not shown). The other genes listed in Figure 2 did not interact with GST-Mo25α (data not shown). Similarly, Mst1 and Mst2 did not interact with Gst-Mo25α (Figure 2B), underscoring the specificity of the interaction between Mst4 and Mo25α.

To document an endogenous interaction between Mo25α and Mst4, we induced expression of Flag-tagged Stradα in W4 cells as bait to capture endogenous Mo25α complexes (Figure 2C). This indeed allowed us to precipitate endogenous Mo25α. Moreover, endogenous Mst4 readily coprecipitated with Mo25α (Figure 2C). These proteins were only seen in immunoprecipitations derived from lysates of doxycycline (Dox) -induced cells, confirming the specificity of the interaction. Furthermore, Mst4 did not coprecipitate with Stradα in the absence of endogenous Mo25α (Figure 2D), indicating that Strad does not form a direct interaction with Mst4. These data show unambiguously that endogenous Mo25α is complexed with endogenous Mst4, at least when Mo25α is bound to induced Stradα.

In parallel to the Y2H screen, we performed an in vitro kinase assay with 80 selected kinases on an Ezrin peptide containing the T567 phosphorylation site (Figure 3A). In this screen, we identified the Mst and Rock kinases as the most potent kinases for the Ezrin peptide (Figure 3A). The latter kinase is a well-known activator of Ezrin in cell migration (Matsui et al., 1998; Haas et al., 2007; Lee et al., 2004; Sahai and Marshall, 2003). Although Mst1 and 2 were also able to phosphorylate Ezrin, they do not interact with GST-Mo25α (Figure 2B). As a result of these findings, we therefore excluded Mst1 and 2 from further studies. Recently, PKCi was also identified as an Ezrin kinase (Wald et al., 2008). However, PKCi did not show any activity on the Ezrin peptide in our hands (Figure 3A), whereas it readily phosphorylated CREB (S133) and MARCKS(S152/156) -derived peptides (data not shown).

To confirm Mst4's specificity for Ezrin, we performed in vitro kinase assays on a set of 80 selected peptide substrates (Figure 3B; see Figure S1 available online). Significant levels of phosphorylation were only observed with the Ezrin-derived peptide, suggesting that Mst4 was highly dedicated to Ezrin as its downstream target.





Protein:	Peptide Sequence:	Antibody:	
Ezrin	562-RDKYKtLRQIR-572	anti-Ezrin	pT567
eIF4G	1101-VVQRSSLsRERGE-1113	anti-eIF4G	pS1108
p90RSK	373-HQLFRGFsFVATGLM-387	anti-p90RSK	pS380
p70S6K	382-NQVFLGFtYVAPSV-395	anti-p70S6K	pT389
Bad	150-ELRRMsDEFEG-160	anti-Bad	pS155
C/EBPb	98-AKPSKKPsDYGYVSL-112	anti-C/EBPb	pS105
TH	33-RFIGRRQsLIEDARK-47	anti-TH	pS40
Cdc2	154-GIPIRVYtHEVVTLWY-169	anti-Cdc2	pT161
eIF4E	204-ATKSGsTTKNRFVV-217	anti-eIF4E	pS209

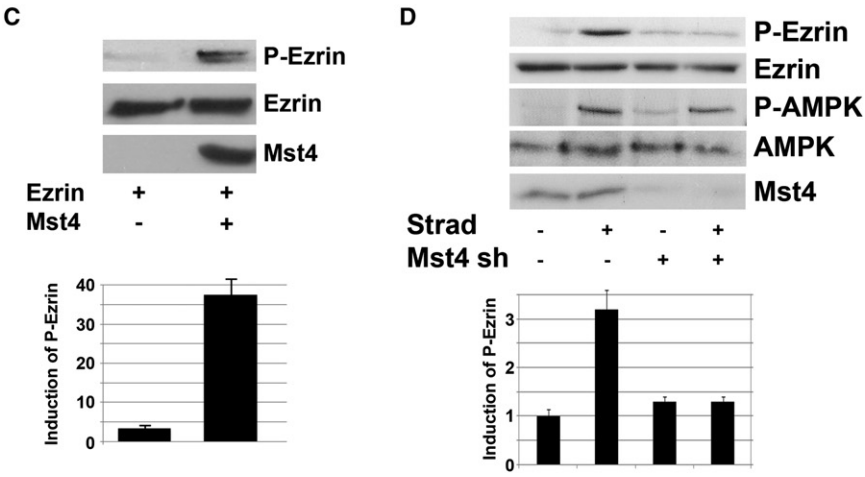


Figure 3. Mst4 Links Lkb1 to Ezrin Phosphorylation

(A) A subset of the 80 kinases tested in a DELFIA-based in vitro kinase assay with an Ezrin-derived substrate peptide representing threonine 567.

(B) A subset of in vitro kinase assays with purified Mst4 on 80 substrate peptides. Phosphorylation was determined by means of the DELFIA technique combined with phospho-specific antibodies.

(C) Mst4 phosphorylates Ezrin in vitro. Purified Ezrin was incubated for 15 min at 37°C in the presence or absence of purified Mst4. Ezrin phosphorylation was visualized after western blotting with a specific antibody raised against phosphorylated Ezrin T567. Quantification was performed by ImageJ (http://rsb.info.nih.gov/ij) and represents three independent experiments. Error bars indicate SD after normalization to a noninduced sample in control cells.

(D) Lkb1-induced phosphorylation of Ezrin is dependent on Mst4. W4-derived lysates, expressing pRetrosuper with shRNA against Mst4 or a scrambled sequence (Scr) and treated with or without doxycycline, were blotted and immunostained for Mst4, AMPK, phospho-AMPK, Ezrin, and phospho-Ezrin. Quantification was performed by ImageJ and represents three independent experiments. Error bars indicate SD after normalization to a noninduced sample in control cells.

To extend these observations, we purified recombinant Ezrin and Mst4 from bacteria and performed an in vitro kinase assay. The samples were subjected to western blotting and the level of phospho-Ezrin was determined with a specific antibody raised against pT567 Ezrin (Figure 3C). These data confirmed that full-length Ezrin can be phosphorylated in the presence of purified Mst4 at the predicted sequence.

To explore whether the phosphorylation of Ezrin was indeed dependent on Mst4 in vivo, we cultured shRNA-mediated Mst4 knockdown cells and scrambled (Scr) controls with or without doxycycline and performed western blot analysis for phosphorylated T567 Ezrin, total Ezrin, and Mst4. Dox-induced activation of Lkb1 resulted in phosphorylation of Ezrin at T567 and, in agreement with the in vitro analysis from Figures 3A–3C, cells lacking expression of endogenous Mst4 lost their ability to induce the phosphorylation of endogenous Ezrin upon Strad induction (Figure 3D). As a control for Lkb1 activity, we stained the western blots for phosphorylated AMPK and total AMPK (Hong et al., 2003) and observed that activation of endogenous AMPK by Lkb1 was independent of Mst4 (Figure 3D).

Mst4 and Ezrin Act Downstream of Lkb1 in Brush Border Formation

The potential role for Mst4 in polarization was tested by shRNA-mediated knockdown in W4 cells. The cells were seeded in the presence or absence of doxycycline for 24 hr in order to induce polarization (Baas et al., 2004). Subsequently, the cells were either stained for actin (Figure 4A) or tested for expression of Mst4, Stradα, Lkb1, and Mo25α on western blot (Figure 4B). In control cells, we observed a clear induction of brush border formation (46/50 cells) (Figure 4A), whereas brush borders were virtually absent in Mst4 knockdown cells (4/50). Western blot analysis showed no reduction in Lkb1, Strad, or Mo25α, indicating that the effect was not due to reduced expression of these proteins (Figure 4A). Importantly, exogenous expression of Myc-tagged mouse Mst4 in Mst4 knockdown cells rescued brush border formation

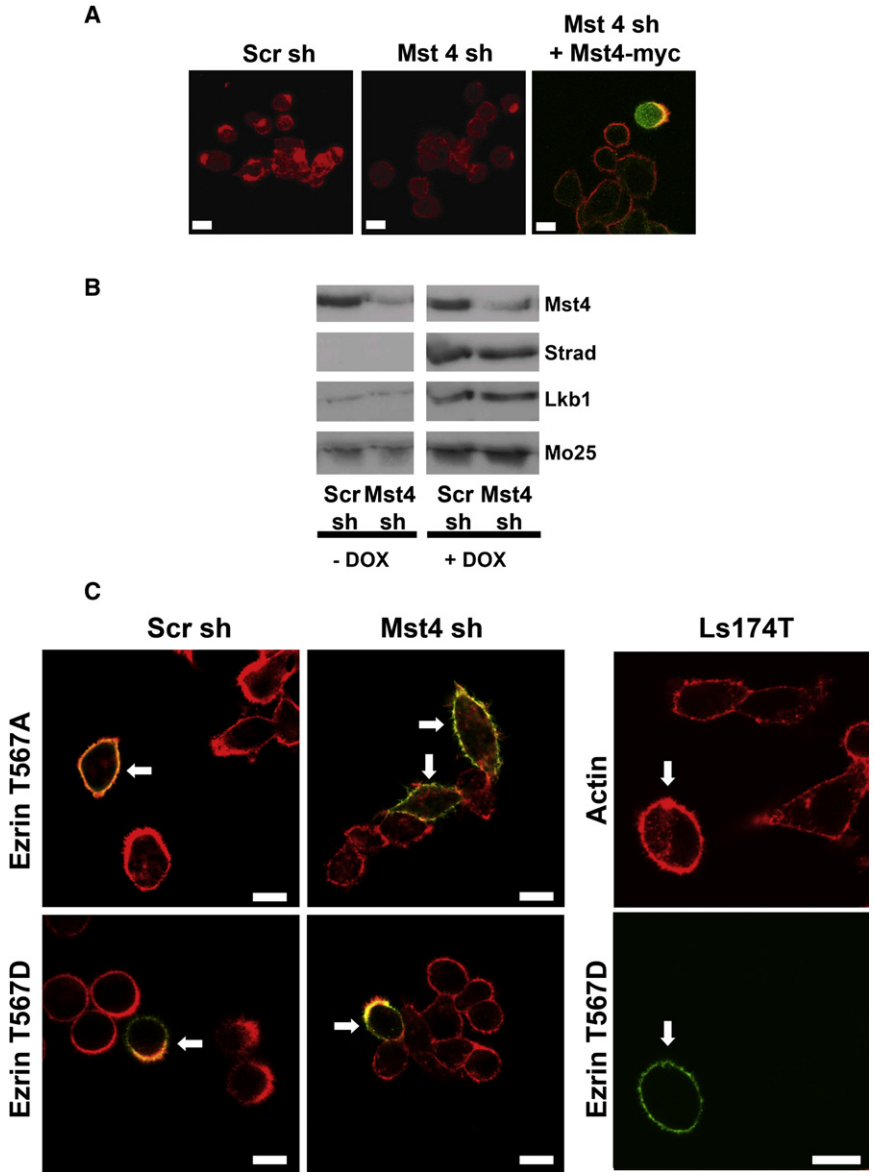


Figure 4. Lkb1 Induces Brush Borders via Mst4 and Ezrin

(A) Reduced expression of Mst4 in W4 cells results in loss of polarization. W4 cells with shRNA against Mst4 or a scrambled sequence (Scr) with or without exogenous expression of wild-type Mst4 were treated with doxycycline for 24 hr, fixed, and immunostained for actin (red) and Myc-tagged Mst4 (green). Arrows indicate brush borders. The scale bars represent 10 μm.

(B) Protein expression of Mst4, Strad, Lkb1, and Mo25α in W4 cells expressing shRNA for Mst4. W4-derived lysates expressing pRetrosuper with shRNA against Mst4 or a scrambled sequence (Scr) and treated with or without doxycycline were blotted and immunostained for Mst4, Strad, Lkb1, and Mo25α.

(C) Ezrin acts downstream of Mst4 in brush border formation. Ls174T and W4 cells with shRNA against Mst4 or a scrambled sequence (Scr) with or without exogenous expression of EzrinT567A or T567D were treated with doxycycline for 24 hr, fixed, and immunostained for actin (red) and VSV-tagged Ezrin (green). Arrows indicate transfected cells. The scale bars represent 10 μm.

(44/50), thereby excluding off-target effects as a result of the shRNA transfection (Figure 4A). These data indicated that Mst4 mediates Lkb1-induced brush border formation of Ls174T cells.

To establish that shMst4 cells were unable to form brush borders as a result of their inability to activate Ezrin, we expressed inactive (T567A) and active (T567D) Ezrin in W4 cells. Cells were stained for VSV-tagged Ezrin mutants and brush borders were visualized by phalloidin-rhodamine (Figure 4C). Confocal analysis revealed that shMst4 cells expressing the active Ezrin T567D regained their ability to form brush borders (41/50) (Figure 4C), whereas the T567A Ezrin mutant-expressing cells did not form brush borders (0/50) (Figure 4C). From this, we concluded that Ezrin phosphorylation acts downstream of the Lkb1-Mst4 pathway in brush border formation. Moreover, expression of active Ezrin in either Ls174T cells (Figure 4C) or nonpolarized W4 cells (data not shown) results in the formation of nonpolarized brush borders (41/50). This suggests that Lkb1 regulates both the polarized localization and Mst4-dependent activation of Ezrin.

Lkb1 Regulates the Localization, but Not Autophosphorylation, of Mst4

The Mst4 serine/threonine kinase is autophosphorylated at T178, an essential prerequisite for full kinase activity, because mutation of T178 into alanine (T178A Mst4) results in a dominant-negative mutant of Mst4 (Preisinger et al., 2004). This mutant was used to test the requirement for the kinase activity of Mst4 in brush border formation. Myc-tagged wild-type and T178 Mst4 were transfected into W4 cells and subsequently seeded in the presence of doxycycline. Cells were stained for the Myc-tagged Mst4 proteins together with actin. Confocal imaging showed that cells expressing the T178A Mst4 protein lost their ability to form brush borders, whereas the WT Mst4-expressing cells formed brush borders in a normal fashion (Figure 5A).

The major mechanism by which Lkb1controls the activity of downstream MARK/AMPK family kinases involves the phosphorylation of residues in their regulatory loop. In order to analyze whether Lkb1 may control the activity of Mst4 by a similar mechanism, we generated W4 cells expressing HA-tagged Mst4 and immunoprecipitated Mst4 from these cells treated with doxycycline for 24 hr or with 10 mM NaF for 30 min. Of note, NaF is a nonspecific and cell-permeable Ser/Thr phosphatase inhibitor which will increase the phosphorylation of a large number of proteins in living cells (Jaumot and Hancock, 2001). Western blot analysis was performed with antibodies against Mst4 and T178 phospho-Mst4 (Figure 5B). Although Lkb1 is fully

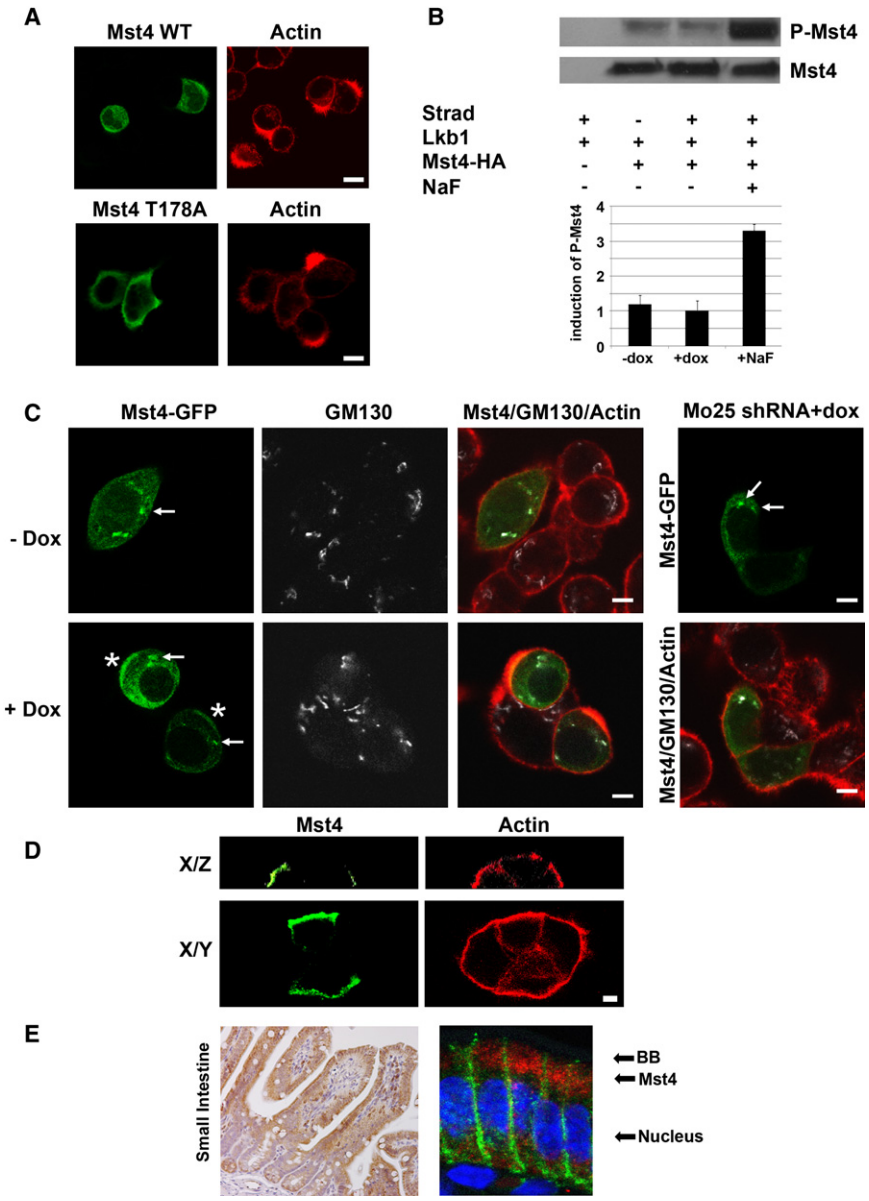


Figure 5. Lkb1 Regulates the Localization of Mst4

(A) Expression of kinase inactive Mst4 in W4 cells results in loss of polarization. W4 cells with wild-type (WT) Mst4 or kinase inactive (T178A) Mst4 were treated with doxycycline for 24 hr, fixed, and immunostained for actin (red) and Myc-tagged Mst4 (green). The scale bars represent 5 μm.

(B) Lkb1 does not regulate the autophosphorylation of Mst4. W4 cells with exogenous expression of HA-tagged Mst4 were subjected to immunoprecipitations with anti-HA in RIPA buffer and subsequently blotted and stained with anti-phospho-Mst4/Ysk1. Quantification was performed by ImageJ and represents three independent experiments. Error bars indicate SD after normalization to a noninduced sample in control cells.

(C) Lkb1 induces the translocation of Mst4 in W4 cells. W4 or W4 shRNA Mo25α cells expressing GFP-Mst4 were treated with doxycycline for 24 hr, fixed, and immunostained for actin (red), GM130 (white), and GFP-Mst4 (green). Asterisks indicate brush borders; arrows indicate vesicles. The scale bars represent 5 μm.

(D) Mst4 localizes to the apical side of DLD-1 cells. DLD-1 cells expressing Mst4-GFP are in green. Actin is indicated in red. The scale bar represents 5 μm.

(E) Mst4 localizes to the apical side of enterocytes in the small intestine. Small intestine was stained for endogenous Mst4; brown color indicates Mst4, and nuclei are stained in blue. Confocal image shows Mst4 (red), E-cadherin (green), and nuclei (blue) in small intestine.

active after a 24 hr induction of Strad (Baas et al., 2003), there was no obvious increase in Mst4 autophosphorylation, suggesting that Lkb1 does not regulate the kinase activity of Mst4.

Another way of regulating proteins is through changes in subcellular localization. Mst4 has been described as a GM130-binding protein localizing to the Golgi apparatus in migrating fibroblasts (Preisinger et al., 2004). To explore the possibility that Lkb1 regulates the localization of Mst4, we expressed Mst4-GFP in W4 cells and induced their polarity with doxycycline. Cells were then fixed and stained for GM130 and actin. Confocal analysis showed that in nonpolarized W4 (50/50) (Figure 5C) and in the parent Ls174T cells (50/50) (not shown), Mst4 was restricted to GM130-containing vesicles similar to the reported localization in fibroblasts (Preisinger et al., 2004). Upon polarization, Mst4 accumulated at the subapical domain of the cell near the brush border, as revealed by phalloidin staining (Figure 5C). The translocation of Mst4 was not observed in the shRNA Mo25α cells (Figure 5C), indicating that the translocation of Mst4 was fully dependent on Mo25α.

In spontaneously polarized DLD-1 cells, we observed a similar apical localization of Mst4 (Figure 5D). Furthermore, we performed an in vivo staining of Mst4 on mouse tissues and could find a specific Mst4 staining in the apical domain of enterocytes in the small intestine (Figure 5E). In other tissues, there was no obvious staining for Mst4. This supports our earlier observation in W4 cells that Mst4 is localized at the apical domain of polarized epithelial cells.

Mst4 Induces Brush Borders, but No Other Polarity Events Downstream of Lkb1

Next, we addressed whether the effect of Mst4 was restricted to brush border formation or, alternatively, extended to other aspects of Lkb1-induced polarity. To that end, sorting of the apical markers CD66 and CD26 and basal TfR were studied in shMst4 and shScr W4 cells. The stainings revealed that both CD66 (45/50) and CD26 (43/50) localized to the apical domain and TfR to the basal site of the cells (39/50) in an Mst4-independent fashion (Figure 6A), whereas the localization of the brush

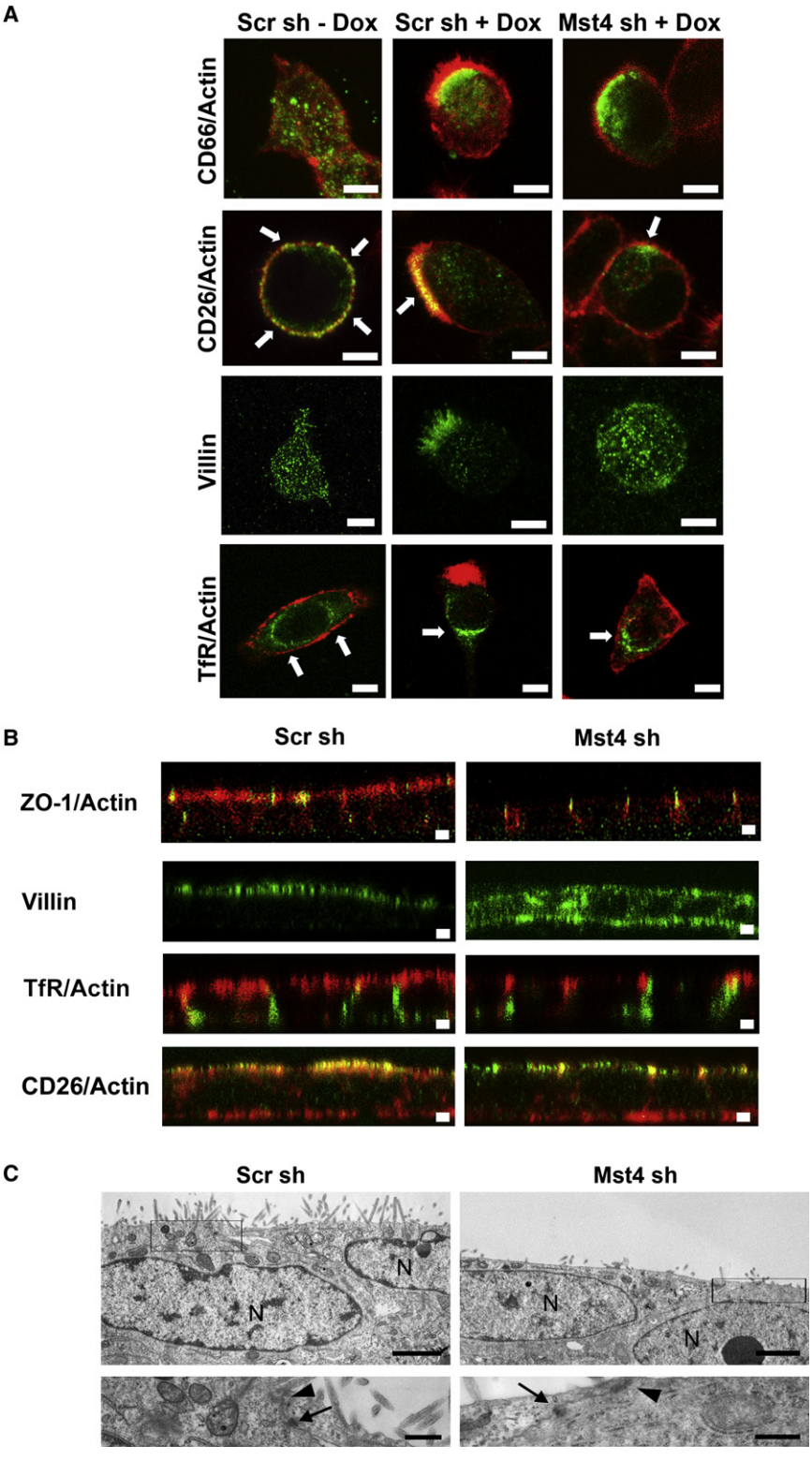


Figure 6. Mst4 Plays a Pivotal Role in Brush Border, but Not Apical, Polarity

(A) Mst4 mediates brush borders. W4 cells with shRNA against Mst4 or a scrambled sequence (Scr) were treated with doxycycline for 24 hr, fixed, and immunostained for actin (red), the apical markers CD66 and CD26 (green), basal marker TfR (green), or brush border marker Villin (green). The scale bars represent 10 μm. Arrows indicate localization of either CD26 or TfR.

(B) Reduced expression of Mst4 in Caco2 cells results in loss of brush borders. Caco cells with shRNA against Mst4 or a scrambled sequence (Scr) were cultured for 3 weeks to allow polarization and differentiation, and subsequently fixed and immunostained for actin (red) and ZO1 (green), Villin (green), TfR (green), or CD26 (green). The scale bars represent 5 μm.

(C) Reduced expression of Mst4 in Caco2 cells results in loss of microvilli. Caco2 cells expressing scrambled shRNA (shSCR) or Mst4 shRNA (shMst4) were cultured for 3 weeks, fixed, and subjected to transmission electron microscopy. Arrows indicate desmosomes; arrowheads indicate tight junctions; N indicates the nucleus in the boxes indicated in the zoomed-in images. The scale bars represent 1 μm.

border marker Villin was fully dependent on Mst4 (43/50) (Figure 6A).

Previously, we have shown that Caco2 cells, a cell line widely used for polarity studies, relies on Lkb1 for its spontaneous polarization (Baas et al., 2004). We therefore explored the physiological relevance of Mst4 for brush border formation in Caco2 cells spontaneously polarized after 3 weeks of culture in a confluent monolayer (Chantret et al., 1988). Analyses were performed by staining for the brush border marker Villin, the tight junction marker ZO1, apical marker CD26, basal marker TfR, and actin. Cells in which Mst4 was downregulated by shRNA had lost the ability to form brush borders, as evidenced by the failure to recruit Villin to the apical site of the cell (Figure 6B). However, polarized localization of ZO1, CD26, and TfR in these cells was not altered (Figure 6B). This suggested that Mst4 mediates brush border formation, rather than polarization per se.

In parallel to the confocal imaging of polarized Caco2 cells, we analyzed the cells by transmission electron microscopy and observed a strong reduction in microvillus length and number when Mst4 expression was reduced by means of shRNA (Figure 6C). Of note, the organization of tight junctions, desmosomes, and cell-cell junctions in shMST4 cells appeared to be unaffected when compared to control Caco2 cells (Figure 6C), thus confirming the conclusion that Mst4 specifically directs the formation of actin-based brush border structures at the apical domain and does not mediate the effect of Lkb1 on polarization of cell-cell junctions.

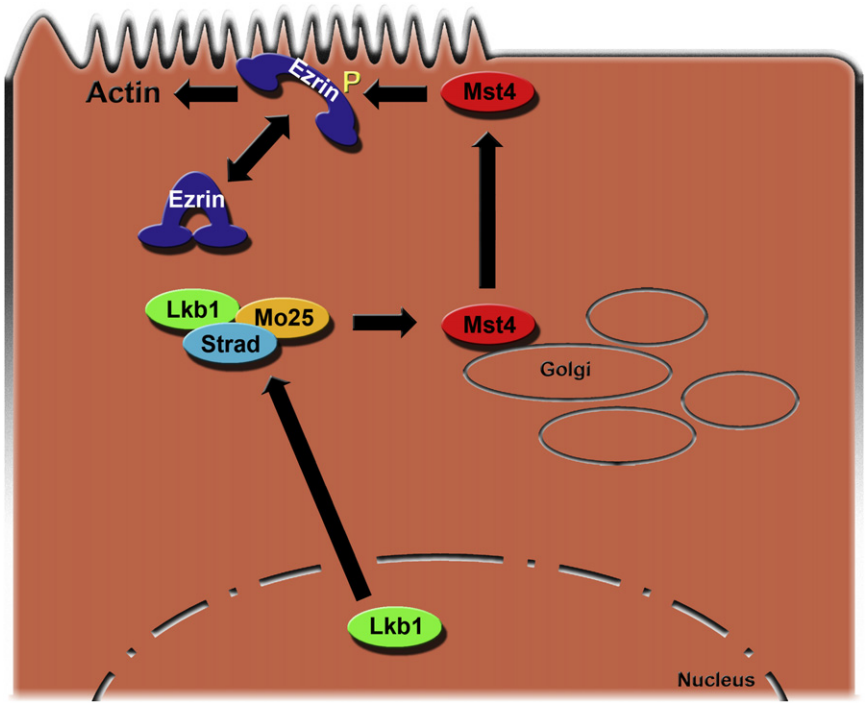


Figure 7. Model
Strad and Mo25 interact and activate Lkb1 in the cytoplasm. This results in the translocation of Mst4 from Golgi vesicles to the apical membrane and the polarized localization of Ezrin. Subsequently, Mst4 phosphorylates and thereby activates Ezrin, which is an essential step in the formation of brush borders at the apical site.

DISCUSSION

During epithelial cell polarization, cells specify their basal, lateral, and apical membrane domains. A controlling role of Lkb1 was previously demonstrated in our single-cell system, in which the activation of Lkb1 by Strad results in the formation of brush borders flanked by the tight junction marker ZO1 and in correct sorting of apical and basolateral markers (Baas et al., 2003). In the current study (Figure 7), we have identified Mst4 as an interactor of the Lkb1/Strad/Mo25 complex. Mst4 specifically controls the formation of brush borders at the apical domain and has no effect on polarization per se. Thus, Lkb1 may regulate different aspects of polarity through separate pathways, of which the Mst4-Ezrin pathway is one.

The role of a Mo25-Mst4 interaction in the control of polarity appears conserved between yeast and human. Nak1 is an *S. pombe* S/T kinase and is the closest yeast homolog of the mammalian Mst kinase family. *S. pombe* displays a cylindrical morphology during vegetative growth, characterized by F-actin accumulation and cell growth at one cell tip during the early phase of the cell cycle, whereas forming two growth tips at a later stage (Mitchison and Nurse, 1985). Control of this mono- and bipolar phenotype is exerted by a complex of at least four proteins: Mo25, Nak1, Orb6, and Mor2 (Kanai et al., 2005; Mendoza et al., 2005). Nak1 is at the center of the complex by forming direct physical interactions with Mo25, Orb6, and Mor2 (Kanai et al., 2005). The complex plays a pivotal role in single-cell polarity and actin polymerization at the growing cell tip. Temperature-sensitive inactivating mutations of these genes result in spherical growth (Kanai et al., 2005; Mendoza et al., 2005). It is of interest that the *S. pombe* Mst homolog Nak1 interacts with the *S. pombe* Mo25 homolog to control polarized actin cap formation, a structure that could be considered reminiscent of actin-based brush borders in human epithelial cells.

In *Dictyostelium discoideum*, the Mst family is represented by severin kinase (SvkA). SvkA knockout cells are severely defective in aspects of polarity, as indicated by disturbances in cytokinesis and directed slug movement (Rohlfs et al., 2007). In our experiments with Mst4, we only observed an effect on apical brush border formation and noticed no defects in cytokinesis or migration (data not shown). However, reduced expression of Ysk1/Mst5 and Mst1 in migrating cells reportedly affects polarized migration (Preisinger et al., 2004; Katagiri et al., 2006), suggesting that individual Mst kinase family members might control different aspects of polarity-related cell behaviors.

Mst4 interacts directly with the Golgi matrix protein GM130 (Preisinger et al., 2004). Translocation to the cell membrane is dependent on activation of Lkb1 and appears to be required for the induction of brush borders. We do not know the exact molecular mechanism underlying this regulated translocation event. In migrating cells, reducing the expression of Lkb1 attenuates the Golgi reorientation toward the direction of migration and inhibits the polarized migration of these cells (Zhang et al., 2008). The best-studied kinase downstream of Lkb1 is AMPK. Recent studies show that AMPK can mediate specific polarity signals (Lee et al., 2007; Mirouse et al., 2007). A possible mechanism for AMPK in polarity might, similar to Lkb1, be its effect on the assembly and disassembly of the Golgi (Miyamoto et al., 2008). The notion that vesicle transport is an essential step in polarity has also been illustrated by the recent observation that clathrin mediates basolateral polarity by regulating the exit of basolateral proteins from the Golgi complex (Deborde et al., 2008).

In this paper, we identify Ezrin as a downstream target of Mst4. Phosphorylation of Ezrin at T567 converts the molecule from a closed to an open state, enabling the simultaneous binding of the actin cytoskeleton and the plasma membrane (Niggli and Rossy, 2008). Ezrin can also recruit GEFs for different GTPases, thereby enhancing processes essential for brush border formation, such as actin polymerization (D'Angelo et al., 2007). The role for Ezrin and Ezrin-binding protein 50 in brush border formation has been demonstrated by gene knockout (Morales et al., 2004; Saotome et al., 2004). Both knockouts had a strongly reduced length and number of microvilli in the intestine (Morales et al., 2004; Saotome et al., 2004). Moreover, the observation that active Ezrin-induced brush borders were polarized in Strad-induced cells, whereas they were uniformly

formed in cells without Strad expression (Figure 4C), suggests that Lkb1 also regulates the localization of Ezrin. Moreover, polarized localization of active Ezrin is still observed in shMst4 cells, suggesting that Mst4 is only involved in phosphorylation of Ezrin and not the polarized recruitment of Ezrin to the apical domain (Figure 4C).

In conclusion, our data indicate that Lkb1 can regulate specific polarization events by signaling toward different effector pathways. One of these pathways includes the translocation of Mst4 to the apical domain and, subsequently, localized Ezrin phosphorylation resulting in the formation of brush borders.

EXPERIMENTAL PROCEDURES

Antibodies and Constructs

Antibodies: anti-HA-tag (12CA5) was from Boehringer Mannheim. Anti-Myc tag (sc-789) was obtained from Santa Cruz Biotechnology. Anti-Flag (F1804) was obtained from Sigma-Aldrich. Anti-Mo25 (2027-1) and anti-Mst4 (2049-1) were obtained from Epitomics. Anti-Strad has been described in Baas et al. (2004). F-actin was stained with rhodamine-labeled phalloidin (Molecular Probes).

Plasmids: GST-Mo25, Myc-Lkb1, and Flag-Strad have been described previously (Baas et al., 2004). Mst4 was a kind gift from Francis Barr (Preisinger et al., 2004), pRetrosuper was from Dr. R. Bernards (Brummelkamp et al., 2004), and WT/T567A Ezrin was from Dr. M. Arpin (Fievet et al., 2004). Mst4 mutants were generated by PCR and cloned in a Myc-tagged pCDNA3.1 vector and in GST-6P-1 (27-4597-01; Amersham Biosciences) using the BamHI and Xbal restriction sites. The following primers were used to generate the different Mst4 mutants:

MST4h-1 5′-AAGAATTCATGGCCCACTCGC-3′
MST4h-2 5′-TTTCTAGAAAATGAATTTGTGTTTC-3′
MST4h-3 5′-AAGAATTCATGGTAAAAAATTCAAAG-3′
MST4h-4 5′-TTTCTAGAACTCGGAATCAGATTC-3′
MST4h-5 5′-TTTCTAGAAGGGGGATTCG-3′.

Full-length Mst4 was amplified with primers 1 and 5, kinase-GM130 domains with 1 and 4, kinase with 1 and 2, and GM130-Cterm with 3 and 5.

For the knockdown studies of Mst4, we cloned the following primers in pRetrosuper:

5′-GATCTGGTCTGGACTATCTGCATTTTCAAGAGAAATGCAGATAGTCCAGACCTTTTTA-3′
5′-AGCTTAAAAAGGTCTGGACTATCTGCATTTCTCTTGAAAATGCAGATAGTCCAGACCA-3′.

As control, we used nonfunctional scrambled sequences, as follows:

5′-GATCTTGAGAGTGGTGATCCATCTTTCAAGAGAAGATGGATCACCACTCTCATTTTTA-3′
5′-AGCTTAAAAATGAGAGTGGTGATCCATCTTCTCTTGAAAGATGGATCACCACTCTCAA-3′.

For the knockdown studies of Mo25α, we cloned the following primers in pRetrosuper:

5′-GATCTGCAGTAGCTCAACTTGCTCTTCAAGAGAGAGCAAGTTGAGCTACTGCTTTTTA-3′
5′-AGCTTAAAAAGCAGTAGCTCAACTTGCTCTCTCTTGAAGAGCAAGTTGAGCTACTGCA-3′

and

5′-GATCTCTCCTACTGTTGAATACATTTCAAGAGAATGTATTCAACAGTAGGAGTTTTTA-3′
5′-AGCTTAAAAACTCCTACTGTTGAATACATTCTCTTGAAATGTATTCAACAGTAGGAGA-3′.

Cell Culture

The HEK293, Caco, and W4 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker) containing 10% heat-inactivated fetal calf serum (FCS; GIBCO) at 37°C/5% CO_2. Cells were passaged by trypsinization. HEK293 and W4 cells were transiently transfected with P-PEI (Polyscience); 2 μg of DNA was mixed with 6 μl of P-PEI (1 μg/μl) in 100 μl of DMEM, and incubated for 30 min at room temperature. Subsequently, 2 ml of DMEM with FCS containing 500,000 cells was added to the DNA-PEI mixture, which was then incubated in a six-well plate for 24 hr. W4 cells with stable expression of pRetrosuper-siMst4 or -siMo25α were generated by retroviral transduction and subsequent selection with puromycin 0.5 mg/ml (Invitrogen). Transfection and production of amphotropic retroviruses and pRetrosuper are described elsewhere (Berns et al., 2004; Michiels et al., 1995).

Yeast Two-Hybrid Screen

cDNA encoding full-length human MO25 was cloned into the pB38 vector derived from pBTM116 and used as bait to screen a random primed human placental cDNA library cloned into the pP6 plasmid derived from the original pGADGH. Using a mating approach as previously described (Formstecher et al., 2005), the yeast two-hybrid screen was performed to ensure a minimum of 50 million interactions tested.

Pull-Down Assays

To assay binding of Mst4 to Mo25α, 10×10^6 HEK293 or W4 cells were seeded 1 day prior to the experiment. Cells were lysed in lysis buffer A (50 mM Tris-HCl [pH 7.5], 100 mM NaCl, 10 mM $MgCl_2$, 10% glycerol, and 1% NP40), and centrifuged for 10 min at 14,000 rpm, at 4°C. The supernatant was incubated with the indicated antibody or GST-Mo25 protein (5 μg) in the presence of protein G-coated or GSH-coated beads, respectively (Sigma) at 4°C for 1 hr while rotating. Beads were washed five times in lysis buffer A and resuspended in 20 μl of SDS sample buffer. Protein complex formation was determined by western blot analysis.

GST fusion proteins were purified from BL21 bacteria. Following overnight culture, protein expression was induced with IPTG (0.1 mM, 4 hr, 37°C). Bacteria were centrifuged and resuspended in PBS, 1% Triton X-100, and 10% glycerol and lysed by sonication (2 × 30 s, duty cycle 50% and output 6, Branson sonifier 250). Lysates were cleared by centrifugation for 15 min at 14,000 rpm, at 4°C. GST fusion proteins were isolated by GSH-coated beads while rotating head over head at 4°C for 30 min. Samples were then washed five times with lysis buffer A and used as indicated (2 μg of GST fusion protein per pull-down).

Confocal and Phase Contrast Microscopy

Forty-eight hours after transfection, cells were fixed by 3.7% formaldehyde (Merck) in PBS for 5 min and permeabilized with 0.5% Triton X-100 in PBS. Immunostainings were performed at 37°C for 1 hr with the indicated antibodies. Fluorescent imaging was done with a Leica confocal laser scanning microscope.

Electron Microscopy Fixation and Preparation

Confluent Mst4 shRNA and Scr shRNA Caco cells were fixed in 2% glutaraldehyde, 0.25% $CaCl_2$, 0.25% $MgCl_2$, and 0.1 M Na cacodylate (pH 7.4) at room temperature for 4 hr. After rinsing in Na cacodylate (pH 7.4) at 4°C, cells were postfixed with 1% OsO_4 and 1.5% $K_3Fe(III)(CN)_6$ in 0.065 M Na cacodylate buffer (pH 7.4) for 2 hr at 4°C in the dark. Dehydration was done with ethanol and the cells were flat embedded in Epon. Ultrathin sections were cut perpendicular to the coverslip and stained with uranyl acetate and lead citrate.

In Vitro Kinase Assay

Peptide phosphorylation was measured using dissociation-enhanced lanthanide fluorescence immunoassay (DELFIAÆ) per the manufacturer's instructions (Perkin Elmer). Specific antibody against phospho-Ezrin (T567) was from Cell Signaling Technology (3141). Lanthanide-labeled secondary antibodies were from Perkin Elmer. Fluorescence was analyzed using the DELFIA Victor 1420 multilabel counter and its accompanying software. (DELFIAÆ is a registered trademark of Perkin Elmer.) Varying amounts of kinase were added to a reaction volume of 50 μl containing 1.5 μM biotinylated substrate peptide in streptavidin-coated 96-well plates. Reactions proceeded for

30 min at room temperature. Excess reagent was removed by washing the plate, and phosphorylated peptides were detected using unlabeled anti-phospho-Ezrin primary antibodies and lanthanide-labeled secondary antibodies. Acidic enhancement solution was added per the manufacturer's instructions, to dissociate the lanthanide ions from the antibody, and stable fluorescent lanthanide chelates were quantified by time-resolved fluorescence.

SUPPLEMENTAL DATA

Supplemental Data include one figure and can be found with this article online at http://www.cell.com/developmental-cell/supplemental/S1534-5807(09)00044-6.

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