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Complete Polarization of Single Intestinal Epithelial Cells upon Activation of LKB1 by STRAD

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Summary

The *LKB1* gene encodes a serine/threonine kinase that is mutated in the Peutz-Jeghers cancer syndrome. LKB1 is homologous to the Par-4 polarity genes in *C. elegans* and *D. melanogaster*. We have previously reported the identification and characterization of an LKB1-specific adaptor protein, STRAD, which activates LKB1 and translocates it from nucleus to cytoplasm. We have now constructed intestinal epithelial cell lines in which inducible STRAD activates LKB1. Upon LKB1 activation, single cells rapidly remodel their actin cytoskeleton to form an apical brush border. The junctional proteins ZO-1 and p120 redistribute in a dotted circle peripheral to the brush border, in the absence of cell-cell contacts. Apical and basolateral markers sort to their respective membrane domains. We conclude that LKB1 can induce complete polarity in intestinal epithelial cells. In contrast to current thinking on polarization of simple epithelia, these cells can fully polarize in the absence of junctional cell-cell contacts.

Introduction

Peutz-Jeghers Syndrome (PJS) patients are clinically characterized by mucocutaneous pigmentation, the development of hamartomas in the gastrointestinal tract, and a predisposition to rare types of cancer (Peutz, 1921; Jeghers et al., 1949; Giardiello et al., 2000). The tumor suppressor protein LKB1 is a serine/threonine kinase, which is mutated in the germline of the large majority of PJS patients (Hemminki et al., 1998; Jenne et al., 1998). Lkb1$^{+/-}$ mice develop gastrointestinal polyps and hepatocellular carcinomas, confirming its role as a tumor suppressor (Bardeesy et al., 2002; Jishage et al., 2002; Miyoshi et al., 2002; Nakau et al., 2002; Rossi et al., 2002). LKB1 is essential during mouse development: Lkb1$^{-/-}$ mice die at midgestation, displaying a variety of developmental abnormalities, including in the yolk sac and placenta (Ylikorkala et al., 2001; Jishage et al., 2002). LKB1 has been proposed to be involved in the control of cell cycle arrest (Tiainen et al., 1999), p53 mediated apoptosis (Karuman et al., 2001), Wnt signaling (Spicer et al., 2003; Ossipova et al., 2003), TGF-β signaling (Smith et al., 2001), ras-induced cell transformation (Bardeesy et al., 2002), and energy metabolism (Hawley at al., 2003; Woods et al., 2003).

Genetic studies in *C. elegans* and *Drosophila* have indicated an essential function of putative LKB1 homologs (Par-4 and dLKB1, respectively) in the establishment of cell polarity (Watts et al., 2000; Martin and St Johnston, 2003). This may suggest a model in which human LKB1 loss causes cellular transformation through disruption of epithelial polarity. Studies of LKB1 have been hampered by the fact that LKB1 exhibits weak catalytic activity in vivo and in vitro. We recently reported the identification of endogenous complexes of human LKB1 with two proteins, STRAD and MO25. The STE20-related pseudokinase STRAD binds to the kinase domain of LKB1 (Baas et al., 2003). In doing so, STRAD transports LKB1 from nucleus to cytoplasm, activates the kinase activity of LKB1, and is phosphorylated by LKB1 (Baas et al., 2003). The small, ubiquitous protein MO25 binds to the C terminus of STRAD and further enhances the effects of STRAD on LKB1 (Boudeau et al., 2003).

We have now constructed human intestinal epithelial cell lines in which the induced expression of STRAD activates LKB1. Upon LKB1 activation, single cells rapidly remodel the actin cytoskeleton to form an apical brush border. Several junctional proteins redistribute in a dotted circle surrounding the brush border. Apical and basolateral markers sort to their respective membrane domains. Caco-2 cells in which LKB1 is depleted by means of RNA interference are affected in their spontaneous polarization. These observations imply that the activation of LKB1 leads to the execution of a complete epithelial polarity program in a cell-autonomous fashion in single cells.

Results

Construction of Intestinal Epithelial Cell Lines with Inducible LKB1 Activity

We observed that the expression of LKB1 mRNA and especially of STRAD mRNA is very low to absent in intestinal epithelial cancer cell lines (A.B., unpublished data). In order to study molecular details of activated LKB1, we attempted to reconstruct the in vivo situation by stably expressing recombinant LKB1 to levels observed in other cell sources. As recipient cell lines, we chose LS174T, a P53 wild-type, E-cadherin mutant colon epithelial cell line, and DLD-1, an E-cadherin wt, P53 mutant colon epithelial cell line. While DLD-1 cells display some degree of polarity when grown to confluency, LS174T cells essentially stay unpolarized under all cell culture conditions, presumably due to their inability to form junctional complexes.

We transfected these cells with a tetracycline (tet)-

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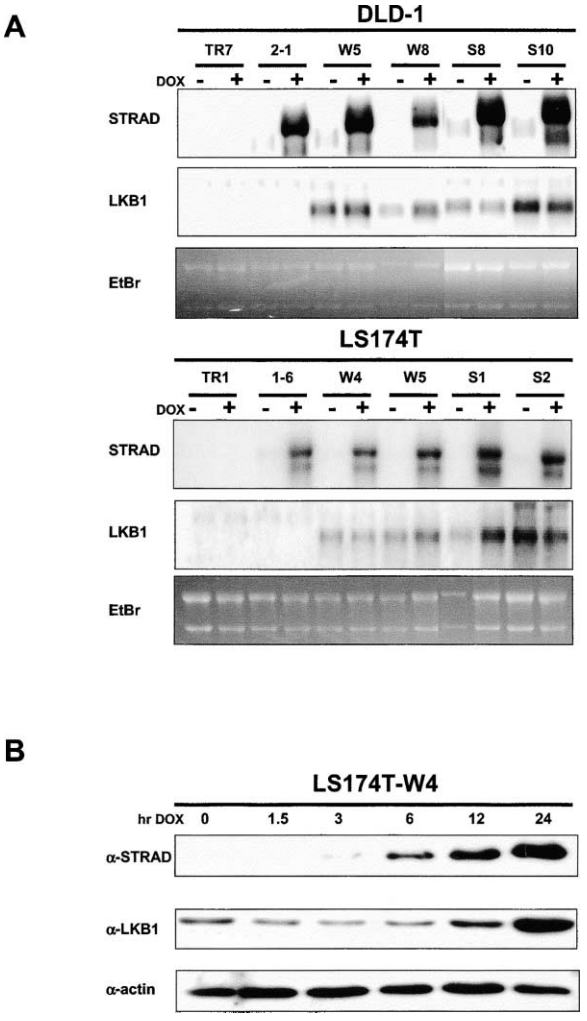

Figure 1. LKB1 Protein Accumulates upon Inducible STRAD Expression in Intestinal Cell Lines

(A) Northern blot analysis of LS174T and DLD-1 clones expressing either Tet-repressor alone (TR1 and TR7, respectively), the Tet-repressor and Tet-repressor-controlled STRAD (1-6 and 2-1, respectively), or both these plasmids, and additionally LKB1-WT (W4/W5 and W5/W8, respectively) or LKB1-SL26 (S1/S2 and S8/S10, respectively).

(B) Time course of doxycyclin treatment of the LS174T-W4 clone shows expression of STRAD within 3 hr. LKB1 protein starts to accumulate after 12 hr.

repressor expression construct (van de Wetering et al., 2002) and a tet-repressor-controlled expression vector (pCDNA4) encoding STRAD. Individual colonies were picked and analyzed for inducible expression of STRAD by Northern blotting (Figure 1A). Upon STRAD induction, no changes were noted in morphology or growth characteristics in any of the clones (data not shown). In selected STRAD-inducible clones, stable transfectants were generated expressing either LKB1-WT (myc-tagged or GFP-tagged), or the mutant LKB1-SL26 (myc-tagged). LKB1-SL26 was originally isolated from a PJS family (Hemminki et al., 1998). It encodes a version of LKB1, which contains an intact kinase domain, but carries a 9 base-pair in-frame deletion C-terminal to the kinase domain that abrogates the interaction with STRAD (Baas et al., 2003). As expected, cells expressing any of the three versions of LKB1 remained unchanged in terms of morphology, cell cycle progression, or apoptosis when STRAD expression was not induced (data not shown). As demonstrated in Figure 1A, several clones were identified for each of the combinations. Figure 1B represents a Western blot analysis of the induction of STRAD in one of the LKB1-expressing clones. Expression of STRAD was visible within 3 hr after induction. After 12 to 24 hr, LKB1 protein started to accumulate. This was a consequence of protein stabilization, mediated by STRAD, since mRNA levels were unaffected upon induction (Figure 1A).

Confocal analysis confirmed that LKB1-WT, as well as LKB1-SL26, were predominantly nuclear before induction. The induction of STRAD, itself expressed throughout the cell in the absence of LKB1, led to cytoplasmic translocation of LKB1-WT, but not of the LKB1-SL26 mutant. Movies of GFP-tagged LKB1 which visualize the STRAD-induced translocation and stabilization in both LS174T and DLD-1 cells are provided as Supplemental Movie S1 available at http://www.cell.com/cgi/content/full/116/3/457/DC1.

Activation of LKB1 Reorganizes the Actin Cytoskeleton and Induces the Formation of a Brush Border

We noticed that the induction of STRAD in cells expressing LKB1-WT led to the retraction of cell extensions within hours of induction (Figure 2A). No effects were seen in the first 24 hr with respect to a reorganization of the microtubule cytoskeleton (data not shown). The actin cytoskeleton, as visualized by phalloidin-TRITC, underwent rapid remodeling. Before induction, actin localized to focal adhesions, stress fibers, and to the cell cortex. Upon induction, focal adhesions were broken down, stress fibers disappeared, and the actin accumulated in a "cap" at the top of the cell. Superimposing the phalloidin stain on a Differential Interference Contrast (DIC) image, suggested that internal organelles sorted away from the actin cap as if the cells were attempting to polarize (Figure 2A). In DLD-1 cells, the cap remained above the center of the cell (Figure 2B, left), while in LS174T transfectants the cap was either present at the top of the cells or at one side of the cells (Figure 2B, right; rotating 3D reconstructions are provided as Supplemental Movie S2 available on the *Cell* website). Induction of STRAD in cells not expressing LKB1 or expressing LKB1-SL26 did not result in these changes (data not shown). To quantify the efficiency of the induction of cap formation, we performed a 24 hr time course study with LS174T-W5 cells. After 3 hr of induction when STRAD protein levels are still hardly detectable (Figure 1B), actin caps were already appearing at significant levels. The presence of the cap coincided with STRAD-dependent cytoplasmic translocation of LKB1 (Figure 3A, arrowheads). The large majority of cells responded by 6 hr. Percentages of cells with actin caps were determined at 0, 3, 6, and 24 hr and are given as a bar diagram in Figure 3C. Induction of STRAD in the LKB1-SL26 transfectants did not lead to the formation of actin caps (Figure 3B).

The hairy aspect of the actin caps as visualized by

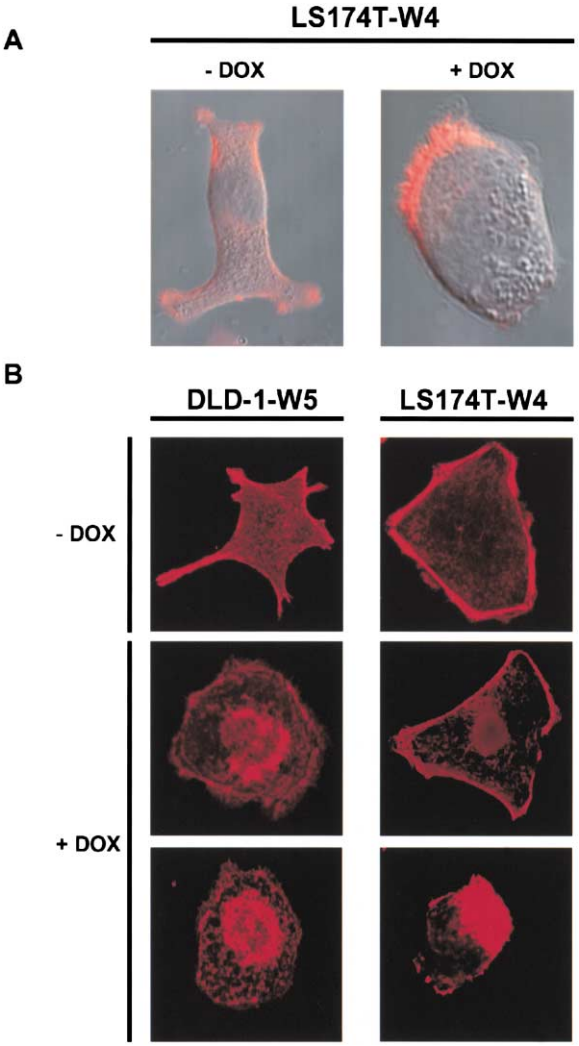

Figure 2. Remodeling of the Actin Cytoskeleton upon Activation of the LKB1/STRAD Complex

(A) DIC image of the LS174T-W4 clone superimposed on a phalloidin-TRITC staining shows retraction of cell extensions and actin cytoskeleton reorganization upon 24 hr doxycyclin treatment. Cell organelles redistribute in a polarized fashion.

(B) Compilations of individual confocal planes show an actin cap on the cell surface of LS174T-W4 and DLD-1-W5 cells after a 24 hr doxycyclin treatment by phalloidin-TRITC staining.

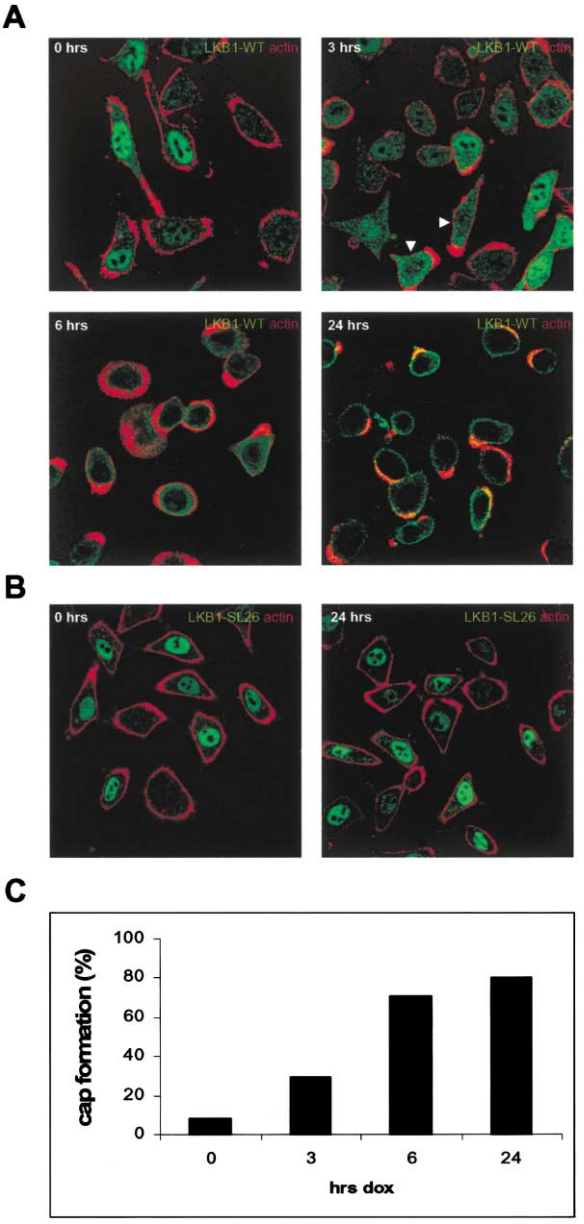

Figure 3. Rapid Actin-Cytoskeleton Remodeling Correlates with LKB1 Translocation

(A) LS174T-W5 cells were subjected to a doxycyclin treatment for the indicated time points. The actin-cytoskeleton is rapidly remodeled in induced LS174T-W5 cells, as analyzed by double staining for actin and myc-LKB1. At the 3 hr time point, the presence of actin caps correlates with cytoplasmic translocation of LKB1 (arrowheads).

(B) The induced LS174T-S2 clone is unable to form actin caps, and LKB1-SL26 resides in the nucleus upon STRAD expression.

(C) Bar diagram of the percentage of LS174T-W5 cells with actin caps after doxycyclin treatment for the indicated intervals.

DIC optics suggested that some of the actin was present in microvilli. Brush borders consist of highly ordered, dense arrays of microvilli covering the apical surface of villus epithelial cells in the intestine. Intestinal cell lines such as Caco-2, or to a lesser extent DLD-1, can form such structures in vitro. However, the only known way to coerce these cell lines into forming apical brush borders involves long-term culture at confluency (Pinto et al., 1983). We asked whether the actin caps represented attempts of single cells to form brush borders. First, we stained LS174T-W4 cells for villin, a brush border protein that interacts with actin (reviewed by Friederich et al., 1990). Forced villin expression in fibroblasts has been shown to lead to the induction of structures resembling brush borders (Friederich et al., 1989). Before induction, villin was localized throughout the cell and did not exclusively colocalize with actin. Upon induction, villin relocalized almost completely to the actin caps (Figure 4A). Second, by cryo-immuno electron microscopy, we were able to demonstrate the presence of microvilli on induced LS174T-W4 cells (Figure 4B). The microvilli localized near an apparent accumulation of actin and them-

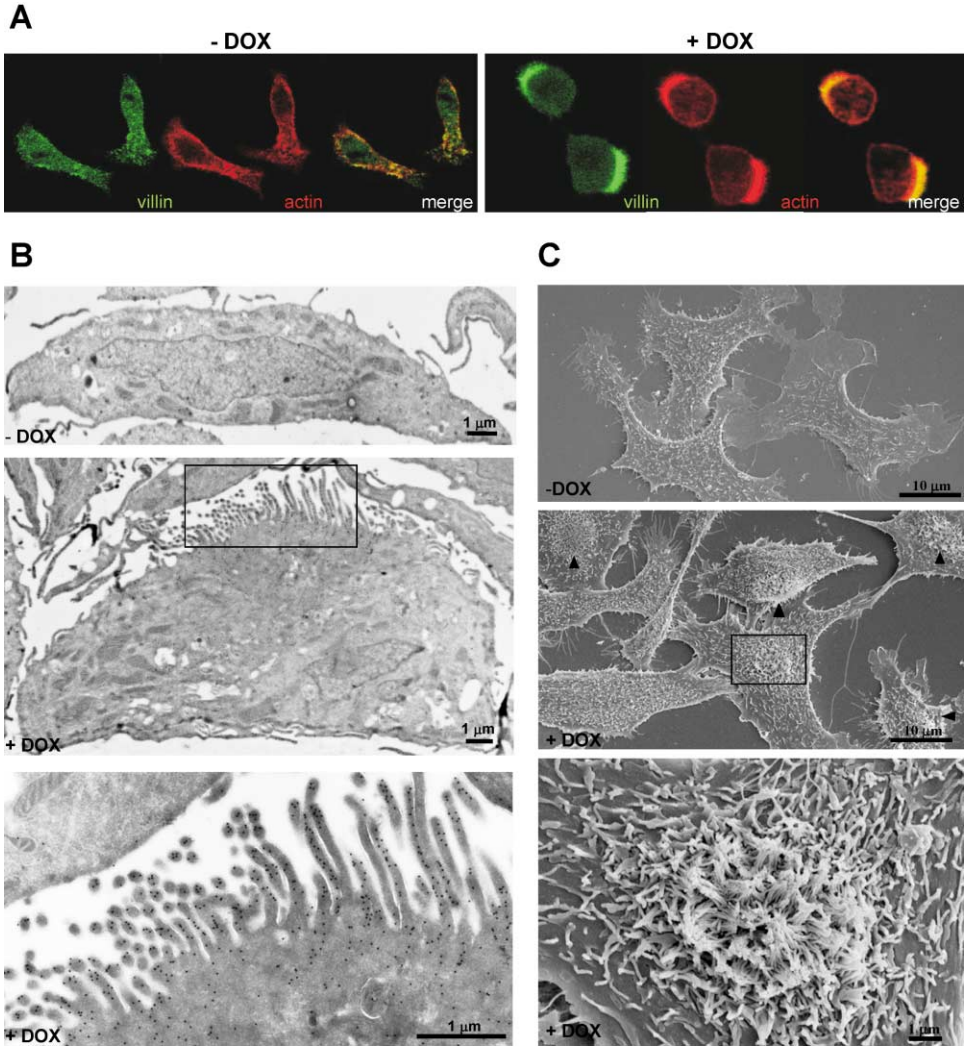

Figure 4. LKB1/STRAD Induces the Formation of Brush Border-Like Structures in Single LS174T-W4 Cells

(A) Double staining of actin and villin reveals strict colocalization of these proteins after a 24 hr doxycyclin treatment (right), while untreated LS174T-W4 cells express villin throughout the cell (left).

(B) Cryo-immuno electron microscopy demonstrates the presence of microvilli in induced LS174T-W4 cells at the sites were actin accumulates. Black dots represent immunogold-labeled actin.

(C) Scanning electron microscopy illustrates the appearance of dense patches of microvilli centered on top of induced LS174T-W4 cells (arrowheads).

selves contained actin, as visualized by immunogold labeling. Third, scanning electron microscopy revealed that cells before induction carried low numbers of scattered, short ruffles of the plasma membrane. Upon induction, a round patch of dense microvilli appeared directly above the center of the cell (Figure 4C). We concluded that LKB1/STRAD can induce the assembly of brush border-like structures in single cells.

Activation of LKB1 Relocalizes Some Junctional Proteins around the Periphery of the Brush Border

A second prominent aspect of polarized simple epithelia is the presence of junctional complexes at the apical boundaries between neighboring cells (reviewed by Fleming et al., 2000). These junctions form an impenetrable seal between cells and provide strength to the epithelial sheet by serving as anchoring sites for cytoskeletal elements including the brush border (reviewed by Zahraoui et al., 2000). We found that LS174T cells do not express junctional proteins, such as ZO-1, and are homozygous mutant for E-cadherin (data not shown). By contrast, DLD-1 cells are capable of forming tight junctions and adhesion junctions when grown to confluency and appear to express most junctional components already at low-cell density. We determined the localization of the tight junction component ZO-1 (Stevenson et al., 1986) and of the adherens junction protein p120 (Anastasiadis and Reynolds, 2000) before and after activation of LKB1 in DLD-1-W5 cells grown at very low density. Before induction, the two proteins were

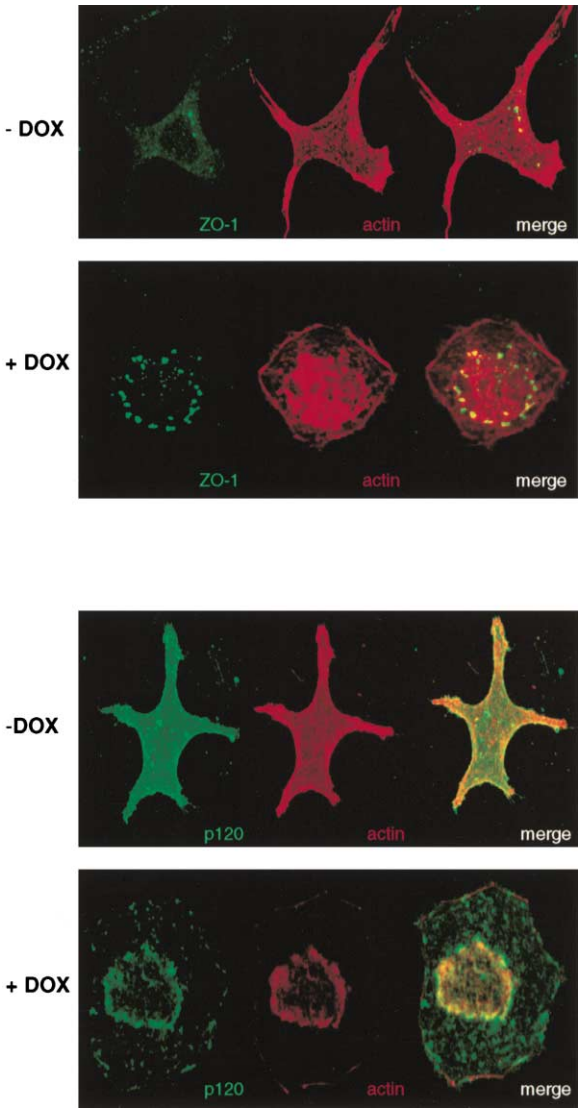

Figure 5. Junctional Proteins ZO-1 and p120 Relocate in a Dotted Pattern Surrounding the Actin Cap

ZO-1 and p120 redistribute in a dotted circle surrounding the actin cap after a 24 hr doxycyclin treatment of DLD-1-W5 cells. This is visualized in compilations of individual focal planes of double staining for actin and ZO-1 (top) and for actin and p120 (bottom).

diffusely expressed intracellularly. Much to our surprise, the proteins relocalized to form a dotted circle directly surrounding the brush border cap (Figure 5). A 3D rotating reconstruction of ZO-1 expression surrounding the actin cap structure is provided as Supplemental Movie S3 available on the *Cell* website. Not all junctional components behaved similarly. E-cadherin and β-catenin localization remained unaffected. Nevertheless, it was evident that at least some junctional proteins were sorted to the appropriate sites to form junctions, i.e., directly adjacent to the brush border, despite the absence of neighboring cells to support cell-to-cell adhesion.

Activation of LKB1 Directs Apical Versus Basolateral Sorting

We next probed a third aspect of polarity in simple epithelia, the sorting of surface proteins to either the apical or basolateral domains. We tested three apical markers, i.e., CD66 (carcinoembryonic antigen/CEA), CD13 (dipeptidyl peptidase IV), and CD26 (amino peptidase-N) that were expressed in LS174T-W4 cells. Before induction of STRAD, all three markers were distributed throughout the cells. Upon induction, all three markers sorted to the apical membrane, although to a somewhat different extent (Figure 6, top). The apical sorting of CD66 was almost absolute. We found the basolateral marker transferrin receptor (CD71) to be expressed in LS174T-W4 cells. The transferrin receptor was present within punctate structures in the cytoplasm and on the cell membrane before induction, consistent with a high rate of cycling through the endocytic route. Upon activation of LKB1 by STRAD induction, the transferrin receptor was strictly excluded from the brush border domain (Figure 6, bottom). These data strongly suggested that LKB1/STRAD-induced polarization results in apical/basolateral sorting of plasma membrane markers.

Activation of LKB1 Induces Polarity in Cell Suspension

To assess whether polarity could be induced independently of contact to a solid support, LS174T-W4 cells were carefully released from culture dishes by EDTA treatment and resuspended as single cells in culture medium. After addition of doxycyclin, the cells were cultured for 4 hr with regular swirling of the tubes to prohibit reattachment of the cells to the plastic. Cytospin preparations were stained for actin, villin, and CD66. Actin caps costaining for villin and CD66 were found to be induced by doxycyclin treatment in this culture system (Supplemental Figure S1 available on the *Cell* website). We concluded that polarity could be imposed by LKB1 and STRAD in the absence of contact to a solid support.

Impairment of Spontaneous Polarization of Caco-2 Cells upon LKB1 Depletion

Lastly, we investigated whether the spontaneous polarization in the classical Caco-2 intestinal cell culture system was also dependent on LKB1 activity. To this end, we targeted five different sequences in the LKB1 gene by shRNA expression constructs in the plasmid pTER (van de Wetering et al., 2003). In transient cotransfection assays with LKB1-myc in HEK293T cells, one of these sequences (#4) was found to efficiently downregulate transfected LKB1 as determined by Western blotting (Figure 7A). The plasmid construct was termed pTER-LKB1. Caco-2 cells were stably transfected with pTER-LKB1 utilizing the selectable G418 marker in this vector. Multiple transfectants were isolated in two independent experiments. LKB1 expression in individual clones was determined by Northern blotting (Figure 7B). Wild-type Caco-2 cells expressed readily detectable levels of LKB1 in concordance with a role for the gene in spontaneous polarization. Different expression levels of LKB1 were observed in individual transfectants ranging from

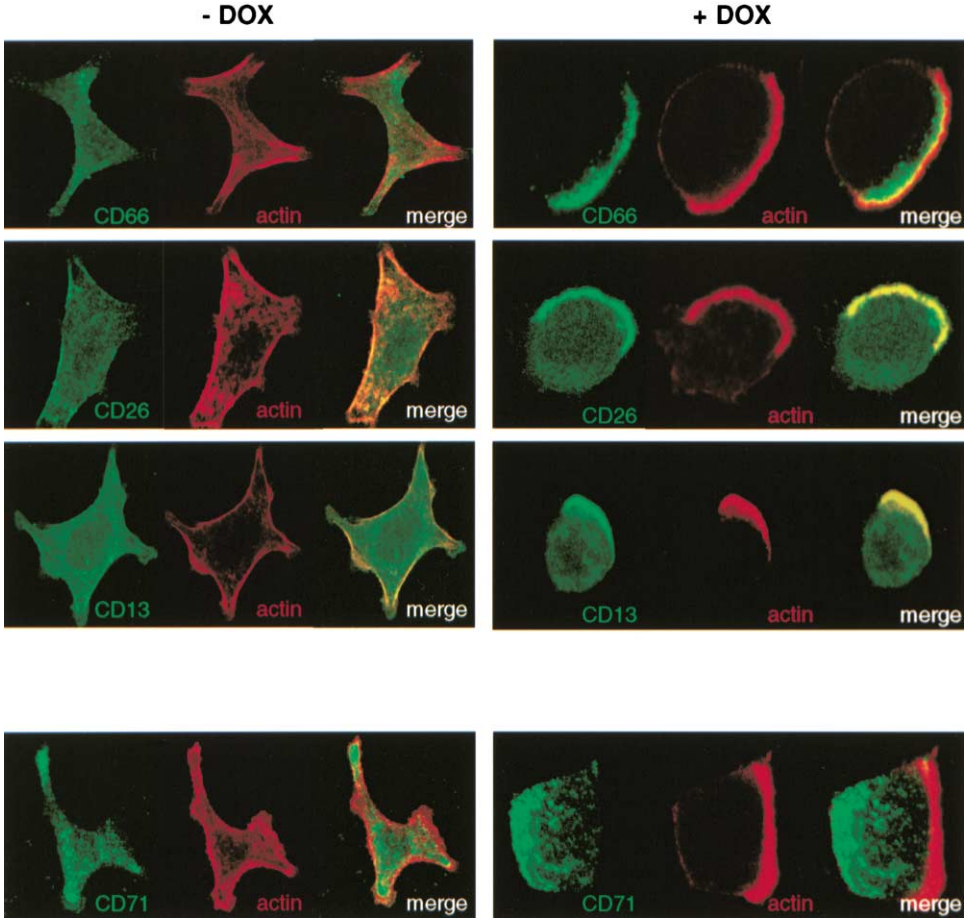

Figure 6. Apical and Basolateral Markers Sort to Their Respective Plasma Domains in Induced LS174T-W4 Cells

Apical and basolateral proteins rearrange to their respective plasma domains in 24 hr doxycyclin-treated LS174T-W4 cells. This is visualized in compilations of individual focal planes of double staining for actin and the apical markers CD66/CD26/CD13 (top) and of double staining for actin and the basolateral marker CD71/transferrin receptor (bottom).

near undetectable (e.g., clone 10) to wt levels (e.g., clone 33). The latter served as a convenient control for the subsequent polarization studies (Figure 7C).

No significant differences in growth characteristics were observed between the shRNA clones and the wt cell line. To further minimize the effects of potential proliferation rate differences in the polarization assay, the clones were seeded at near confluency (i.e., 100,000 cells/cm^2) on glass cover slips and cultured for up to 14 days. At various time points, the cells were fixed and stained to determine the extent of spontaneous polarization. The formation of a continuous apical structure positive for actin, villin, and CD66 was taken as the criterion for polarization. The wt Caco-2 cells, as well as control clone 33, formed a polarized epithelial sheath as determined by staining for actin, villin, and CD66 within 5–7 days after seeding. Clones with a strong knockdown of LKB1 (e.g., 10, 11, 25, 26) were all found to be affected in their ability to polarize. Figure 7C provides representative images of one of the clones (clone 10) compared to wt Caco-2 cells. In some experiments, no polarity was evident after prolonged cell culture (Figure 7C, clone 10, upper images), whereas in other experiments the cells still displayed some tendency to develop foci of polarity, albeit with delayed kinetics (e.g., after 9 days of culturing at confluency; Figure 7C, clone 10, bottom, arrowheads). The remaining ability to polarize to some extent may reflect the inherent limitation of shRNA technology, i.e., that gene knockdown is never complete. However, we cannot exclude from these experiments that the Caco-2 cells may be able to use an alternative molecular mechanism, independent of LKB1, to eventually produce some polarity.

Discussion

In this study, we demonstrate that activation of LKB1 by the induced expression of STRAD polarizes intestinal epithelial cells in a cell-autonomous fashion. Activated LKB1 induced three major aspects of epithelial polarity, i.e., the formation of an apical brush border, the positioning of junctional proteins surrounding this brush border, and the correct sorting of apical and basolateral plasma membrane markers. From our observations, it appears that LKB1 is a bona fide mammalian polarity gene and is thus a genuine ortholog of *C. elegans* Par-4 and *Drosophila* dLKB1. Our data further suggest that orthologs of the STE20-like pseudokinase STRAD could play comparable activator roles in these two model organisms.

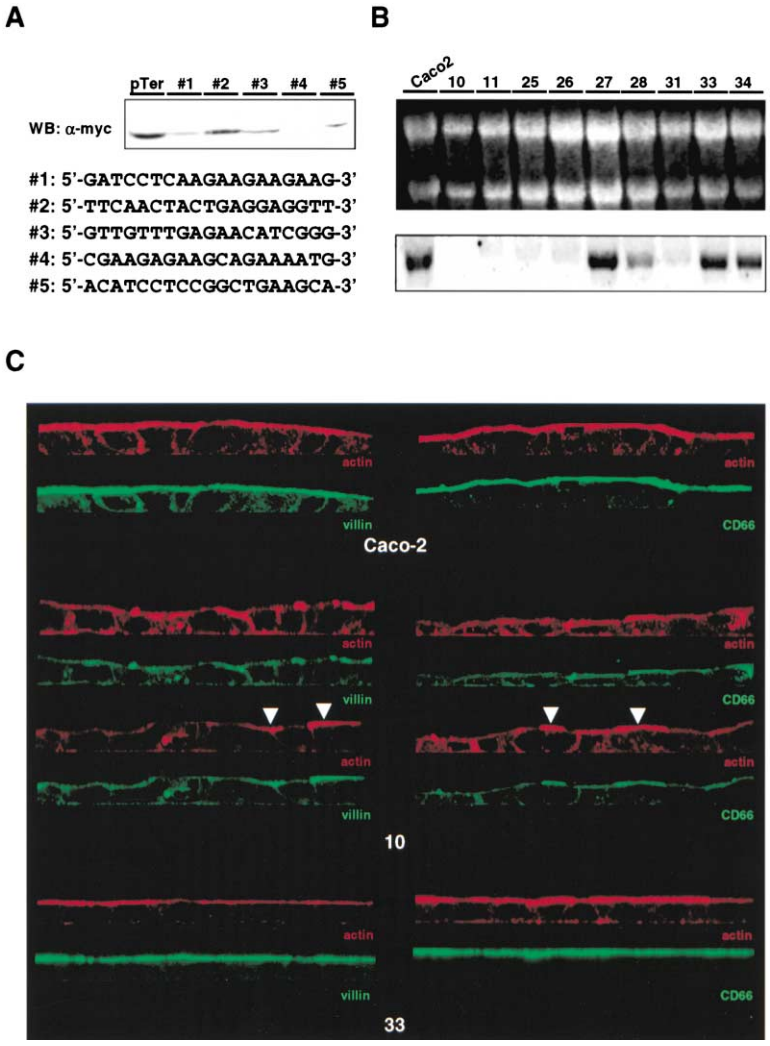

Figure 7. Impairment of Spontaneous Polarization of Caco-2 Cells upon LKB1 Depletion

(A) Five LKB1-specific sequences were targeted by shRNA. Cotransfection assays of the corresponding pTER constructs with LKB1-myc in HEK293T cells reveal that shRNA #4 effectively knocks down LKB1 expression as analyzed by Western blotting.

(B) Northern blot analysis of LKB1 expression in wt Caco-2 and shRNA clones reveals variable LKB1 expression levels.

(C) Vertical optical sections of immunocytochemical analysis of wt Caco-2 cells and clone 10 and 33 at 9 days postconfluency show impaired polarization in clone 10. Wt Caco-2 and clone 33 cells build a continuous brush border containing actin, villin, and CD66. In clone 10, this structure is largely absent (top), although foci of polarity are occasionally observed (bottom, arrowheads).

Indeed, the *Drosophila* genome contains a pseudokinase with high homology to STRAD, which harbors very similar kinase-inactivating amino acid substitutions (NCBI: accession number P83098).

The control of asymmetry of individual cells in multicellular organisms is only beginning to be understood. Cellular polarity involves many independent features, such as an asymmetry of the actin cytoskeleton; the localized distribution of specific mRNAs; the asymmetric localization of the mitotic spindle, of cell junctions, and of intracellular secretory granules; and the sorting of surface molecules to polarized membrane domains. Genetic studies on asymmetric events during early *C. elegans* development have led to the identification of six Par genes (Par-1 through Par-6) that appear to function as master regulators of polarity (Kemphues et al., 1988; Kirby et al., 1990; Morton et al., 1992; Cheng et al., 1995; Guo and Kemphues 1996; Bowerman et al., 1997; Watts et al., 2000). Loss of these Par proteins results in loss of multiple aspects of polarity. Most of the Par proteins in *C. elegans* are themselves distributed in an asymmetric fashion in the early embryo with the notable exception of Par-4, which is diffusely expressed in the cytoplasm and the cellular cortex (Watts et al., 2000). The individual phenotypes of the Par genes are overlapping yet unique (Bowerman et al., 1997).

The absence of gain-of-function PAR phenotypes has hampered epistatic analysis. As a consequence, the connections between the Par proteins have remained largely unknown. It has been proposed that Par-4/LKB1 and Par-1 constitute a kinase cascade, because (1) the two phenotypes are similar; (2) overexpression of Par-4/LKB1 in *Drosophila* partially rescues a Par-1 mutation; and (3) *Drosophila* Par-4/LKB1 is phosphorylated by Par-1 in vitro (Martin and St Johnston, 2003). The phenotype of *Drosophila* LKB1 is of particular interest to the current study. It not only affects the anterior-posterior axis of embryos, but it also disrupts the polarity of the follicular epithelium in mutant clones, a simple epithelium which, like the intestinal epithelium of mammals, normally forms apical adherent junctions (Martin and St Johnston, 2003).

The study of polarity of simple epithelia in mammals has largely been restricted to long-term cultures of epithelial cell lines. These cell lines slowly acquire polarity upon reaching confluency. We are not aware of a cell culture system, in which the induction of epithelial polarity occurs rapidly and in a tightly controlled, cell-autono-

mous fashion. Therefore, the cellular model described here may be a valuable complement to the genetic loss-of-function studies in model organisms. The gain-of-function effects induced by STRAD in our cell lines can be combined with siRNA technology and other approaches to resolve molecular details of the various aspects of polarization of simple epithelia.

One unexpected finding of the current study is the observation that individual epithelial cells can be induced to polarize in the absence of cell-cell contacts, which contradicts current thinking (reviewed by Wodarz, 2000). Previous studies on model cell lines such as MDCK (Cereijido et al., 1980) and Caco-2 (Pinto et al., 1983) have always been restricted to the spontaneous appearance of polarity after the cells are allowed to form cell junctions.

The dynamics of the actin reorganization in our cells suggests that the brush border formation is a direct consequence of LKB1 activation and may be required for the additional polarity phenomena to occur. It appears very likely that the actin reorganization is mediated by Rho family small GTPases. Cdc42 has been found to colocalize with Par-3/-6 (Joberty et al., 2000) and represents a good candidate effector of LKB1-induced polarity. The brush border, once formed, may serve as a landmark to accumulate junctional proteins around its periphery, prior to the assembly of junctional complexes between adjacent cells. The third aspect of epithelial polarity, the definition of apical versus basolateral membrane domains may also directly result from the establishment of the brush border. Possibly, brush border components such as ezrin (Berryman et al., 1993) may specifically anchor and retain apical surface proteins.

It is widely held that tight junctions are crucial to the definition of apical versus basolateral domains, since they serve as an absolute barrier for lateral diffusion of transmembrane proteins (van Meer et al., 1986; Alberts et al., 2002). In this light, it is interesting that our cells do not form cell-cell contacts or junctional complexes (in LS174T), yet are capable of sorting proteins to specific membrane domains.

As has been reported for Par-4 previously (Watts et al., 2000), we never observe LKB1 to be distributed in a polarized fashion. Yet, the actin cap that precedes the formation of a brush border consistently appears directly above the center of the cell. This implies that the cell has spatial cues prior to the induction of STRAD, as it knows where polarized structures should be created. Yeaman et al. (1999) have proposed that cells in simple epithelia and in cell culture dishes derive such spatial cues from integrin signaling occurring at the membrane domain that touches the basal lamina in vivo, or the laminin-coated culture dish in vitro. Of note, the surrogate basolateral domain that is defined by transferrin receptor expression upon LKB1 activation does not coincide with the membrane domain that touches the laminin coat. The former encompasses the entire cellular membrane outside the brush border.

It is currently unknown what controls Par-4/LKB1 activity in the various model systems. cAMP-dependent kinase has been demonstrated to phosphorylate Par-4/LKB1 in *Drosophila* (Martin and St Johnston, 2003) and man (Collins et al., 2000; Sapkota et al., 2001). Our data suggest that the regulated expression of STRAD or the regulated LKB1-STRAD interaction may be a key control point of an LKB1/Par-4 polarity-signaling pathway.

LKB1 has been identified originally as a tumor suppressor. It has since been shown to affect a wide variety of cell growth characteristics (Tiainen et al., 1999, 2002; Marignani et al., 2001; Karuman et al., 2001). Of note, our cell lines do undergo growth arrest upon induction of STRAD (data not shown). This is first observed after 24 hr, long after the cells express the polarized phenotype. When the same cells are induced to undergo a G1 arrest by blocking the Wnt cascade or by inducible expression of cell-cycle inhibitor p21 (van de Wetering et al., 2002), they do not polarize (data not shown). We believe that many of the phenomena that have been attributed to LKB1 may be the indirect consequence of changes in cellular polarity. As suggested previously (Martin and St Johnston, 2003), loss of the tumor suppressor LKB1 may result in cellular transformation as a consequence of disrupted epithelial polarity.

Experimental Procedures

Cell Lines and Transfections

LS174T and DLD-1 cells were cultured in RPMI 1640 media containing standard supplements. LS174T and DLD-1 cells carrying the tet-repressor plasmid (TR1 and TR7, respectively) were generated using the T-rex system (Invitrogen) and were described previously (van de Wetering et al., 2002). Inducible flag-STRAD (pCDNA4) expressing lines were generated according to the manufactures instructions. Resistant clones were tested by Northern blot analysis, according to standard procedures. The STRAD inducible 1-6 (LS174T) and 2-1 (DLD-1) clones were subsequently transfected with LKB1-myc in pCDNA3 (wild-type and SL26 mutant) and LKB1-GFP in pEGFP-C3 (a kind gift from D.R. Alessi and J. Boudeau, University of Dundee, Scotland) using FUGENE 6 reagent (Roche). Transfectants were selected in medium containing G418 (1 mg/ml). Resistant clones were tested by Northern blot analysis.

Western Blot Analysis

Western blotting was performed as described previously (Baas et al., 2003). The following primary antibodies were used: 5c10 (mouse) for LKB1 (see below), 5a11 (mouse) for STRAD (Baas et al., 2003), and antiactin (rabbit) (Sigma).

Generation of LKB1 Monoclonal Antibody (5c10)

A mouse monoclonal LKB1 antibody was generated as described previously (Baas et al., 2003).

Immunocytochemistry

Cells were seeded on laminin (ICN)-coated coverslips (2 $\mu g/cm^2$) at a density of 10,000 cells/cm^2 and were either untreated or subjected to a doxycyclin treatment (1 $\mu g/ml$) for the indicated time points. Subsequently, cells were fixed in 3.7% formaldehyde, followed by permeabilization in 0.1% Triton. The following primary antibodies were used (all mouse, except for CD26): 9E10 (anti-myc) for LKB1, anti-ZO-1, and anti-p120 (both Transduction Laboratories); anti-villin (kindly provided by S. Robine, Institut Curie, France); anti-CD66, anti-CD26 (rabbit) and anti-CD13 (kindly provided by F.X. Real, IMIM, Spain); and anti-CD71-Fitc (Becton and Dickinson). The secondary antibody was FITC-labeled goat-antimouse IgG (SBA) or FITC-labeled goat-antirabbit IgG (Sigma). The actin cytoskeleton was visualized by staining with TRITC-labeled phalloidin (Sigma). Cells were analyzed using a Zeiss LSM510 Meta confocal microscope, enabling 3D compilation of individual confocal planes.

Transmission Electron Microscopy

Cells were seeded on laminin coated coverslips (2 $\mu g/cm^2$) at a density of 10,000 cells/cm^2 and were either untreated or were subjected to a doxycyclin treatment for 24 hr. Subsequently, cells were fixed in 0.2% glutaraldehyde/2% formaldehyde. The collecting, em-

bedding and processing of the fixed cells for cryosectioning with a Leica FCS was performed as described previously (Peters and Hunziker, 2001). Samples were immunogold-labeled using a polyclonal antibody against actin (a kind gift from C. Chaponnier, University of Geneva, Switzerland) and 15 nm gold conjugated to protein-A (EM laboratory, Utrecht University). Sections were analyzed on a Fei CM10 and Tecnai 12 transmission electron microscope.

Scanning Electron Microscopy

Cells were seeded on laminin coated coverslips (2 $\mu g/cm^2$) at a density of 10,000 cells/cm^2 and were either untreated or subjected to a doxycyclin treatment for 24 hr. Subsequently, cells were fixed in 0.2% glutaraldehyde/2% formaldehyde. After fixation, the cells were dehydrated in a graded ethanol series up to 100% and critical point dried under carbon dioxide in a Baltec model CPD 030 critical point drier. The dried specimens were covered with a layer of gold about 3 nm thick in an EMITECH K650X sputter coater and examined at room temperature in a JEOL JSM6700F field emission scanning electron microscope at an accelerating voltage of 2.5 kV.

LKB1 RNA-Interference in Caco-2 Cells

Five LKB1-specific sequences were targeted for RNA-interference by cloning compatible oligonucleotides (Figure 7A, additional sequence information available upon request) into pTER (van de Wetering et al., 2003). For stable transfection, the G418 resistance marker was cloned from pCDNA3 into the pertinent pTER construct. The resulting plasmid was termed LKB1-pTER. Caco-2 cells were transfected with LKB1-pTER and G418-resistant clones were analyzed for LKB1 expression by Northern blotting. To examine the extent of spontaneous polarization, wt Caco-2 cells and LKB1-pTER clones were seeded on laminin-coated coverslips (2 $\mu g/cm^2$) at a density of 100,000 cells/cm^2. Media of the confluent cells was refreshed daily. At various time points postconfluency, morphology was examined and immunocytochemistry experiments for actin, villin, and CD66 were performed as described above.

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