MICAL-like1 mediates epidermal growth factor receptor endocytosis

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Running title: MICAL-L1, a Rab13 effector

Abbreviations: MDCK, Madin Darby Canine Kidney; CH, calponin domain; PRD, proline rich domain; WT, wild type; GST, glutathione S-transferase; EGFR, epidermal growth factor receptor

Abstract

Small GTPase Rabs are required for membrane protein sorting/delivery to precise membrane domains. Rab13 regulates epithelial tight junction assembly and polarized membrane transport. Here, we report that
MICAL-like1 interacts with GTP-Rab13 and shares a similar domain organization with MICAL. MICAL-like1 (MICAL-L1) has a calponin homology, LIM, proline rich and coiled-coil domains. It is associated with late endosomes. Time-lapse video microscopy shows that GFP-Rab7 and cherry-MICAL-L1 are present within vesicles that move rapidly in the cytoplasm. Depletion of MICAL-L1 by short hairpin RNA does not alter the distribution of a late endosomal/lysosomal associated protein, but affects the trafficking of epidermal growth factor receptor (EGFR). Overexpression of MICAL-L1 leads to the accumulation of EGFR in late endosomal compartment. In contrast, knocking down MICAL-L1 results in the distribution of internalized EGFR in vesicles spread throughout the cytoplasm and promotes its degradation. Our data suggest that the N-terminal calponin (CH) domain associates with the C-terminal Rab13 binding domain (RBD) of MICAL-L1. The binding of Rab13 to RBD disrupts the CH/RBD interaction, and may induce a conformational change in MICAL-L1 promoting its activation. Our results provide novel insights into MICAL-L1/Rab protein complex that can regulate EGFR trafficking at late endocytic pathways.

**Introduction**

Epithelial cells exhibit distinct apical and basolateral plasma membranes separated by tight junctions that establish an apico-lateral barrier to prevent intermixing of proteins and lipids between apical-lateral membranes. The maintenance of this apico/basolateral polarity requires sorting and correct delivery of membrane proteins during exocytic and endocytic pathways. Small Rab GTPases regulate various steps of membrane trafficking. They cycle between GTP-bound active and GDP-bound inactive forms. In their active conformation, Rab proteins interact with a variety of effectors to control different cellular processes such as vesicle
sorting/targeting, vesicle movement or kinase activities (Zahraoui et al., 2000; Zerial and McBride, 2001; Tisdale, 2003; Stenmark, 2009). Rab13 is closely related to Rab8, Rab10, and to the yeast Sec4 which is required for polarized transport in yeast (Guo et al., 1999). Rab8, Rab10 and Rab13 regulate surface delivery of membrane proteins to the basolateral side of epithelial cells. In addition, Rab10 also regulates sorting of internalized cargos (Huber et al., 1993; Ang et al., 2003; Babbe et al., 2006; Schuck et al., 2007; Henry and Sheff, 2008; Nokes et al., 2008). Rab13 is recruited to cell-cell contacts from a cytosolic pool at an early stage during the assembly of junctional complexes, suggesting a role for Rab13 in the regulation of cell-cell junctions and/or polarity in mammalian cells (Zahraoui et al., 1994; Sheth et al., 2000). By generating MDCK cell lines expressing either active (Rab13Q67L) or inactive (Rab13T22N) mutants of Rab13, we previously showed that expression of the active, but not the inactive mutant, affects the recruitment of tight junction proteins, claudin1, occludin and ZO-1 (Marzesco et al., 2002). Moreover, we demonstrated that GTP-bound Rab13 directly binds to PKA and inhibits PKA-dependent phosphorylation of vasodilator-stimulated phosphoprotein (VASP), an actin remodelling protein. Activation of PKA blocks the inhibitory effect of Rab13 on the recruitment of VASP, ZO-1, and claudin1 to cell-cell junctions (Kohler et al., 2004). Rab13 has also been implicated in occludin recycling (Morimoto et al., 2005).

To obtain further insight into the role of Rab13, we searched for Rab13 interacting proteins in a yeast two-hybrid screen. We isolated a cDNA encoding MICAL-like1, a human protein that shares similar domain organization to MICAL (Molecule Interacting with CasL). MICAL-like1 (MICAL-L1) interacts with Rab13 in vitro and in vivo. In epithelial MDCK cells, MICAL-L1 is localized to late endosomes and colocalizes with Rab13. Our data indicate that MICAL-L1 is a key regulator of EGF receptor endocytosis in epithelial cells.
Results

**MICAL-L1 interacts with GTP-bound Rab13**

To identify novel targets of the small GTPase Rab13, we used the active form of Rab13 (Rab13Q67L) as a bait in a yeast two-hybrid screen. Eighteen clones were isolated that interacted with Rab13Q67L. Sequence analysis of these clones revealed that they were derived from the same gene and encoded a polypeptide of 300 amino acids that was named RBD (Rab13 Binding Domain). RBD interacted specifically with the wild type (WT) and the active form of Rab13 (Q67L), but not with the inactive mutant Rab13T22N. Furthermore, it did not bind to other small GTPases such as Rab5, Rab6, Cdc42 and Arf6 (Fig. 1A-C). A human full-length cDNA clone was identified that encompassed the RBD sequence and coded for a protein identical to MICAL-L1 (Fig. S1A), MICAL-L1 has a single calponin homology (CH) domain of 102 amino acids, a domain also found in cytoskeletal and signal transduction proteins and known to be involved in actin filament binding (Banuelos et al., 1998). Adjacent to this motif, MICAL-L1 has a LIM domain, a cysteine-rich module found in numerous proteins involved in signal transduction and cytoskeletal organization (Petit et al., 2003). The intermediate part of the protein is a proline-rich domain (PRD) that shares no significant similarity with MICAL or other known proteins. Finally, two putative coiled-coil structures were identified at the COOH terminus of MICAL-L1 within the Rab13 binding domain (RBD). In mammals, there are at least 2 members of the MICAL-like family, MICAL-L1 and MICAL-L2. The homology between these proteins is restricted to the CH, LIM and C-terminal RBD domains (Fig. S1B). Glutathione S-transferase (GST) pull-down experiments were used to confirm the interaction obtained in the two hybrid assay. Fig. 1B shows that GST-RBD only bound Rab13Q67L, but not Rab13T22N. As a control, GST alone did not interact with GFP-Rab13 mutants. Interestingly, GST-RBD also bound Rab7, Rab8, and Rab11, but not Rab4 or GFP (Fig. 1C). Affinity purified antibodies raised against the GST-
RBD fusion protein detected a protein band of apparent relative molecular mass ~116,000 (Mr 116KDa), slightly above the predicted relative molecular mass of MICAL-L1 (Mr 100KDa). This protein band was detected in several mammalian cell lines and in placenta tissue (Fig. 1D). This antibody did not work well by immunofluorescence. We also tested available commercial antibodies and found that they recognized additional bands by immunoblots of MDCK cell extracts (not shown). Our results are in agreement with those of Yamamura et al. (Yamamura et al., 2008) and suggest that MICAL-L1 interacts with GTP-bound Rab13.

**MICAL-L1 localizes to a late endocytic compartment**

The cellular distribution of the GFP-MICAL-L1 was examined by immunofluorescence in epithelial MDCK cells. GFP-MICAL-L1 was detected in vesicle-like structures spread throughout the cytoplasm. A pool of MICAL-L1 was also detected at the cell periphery (Fig. 2A). To compare the distribution of MICAL-L1 with well-characterized markers of intracellular trafficking, we expressed GFP-MICAL-L1 in MDCK cells. GFP-MICAL-L1 was found in vesicular compartments which did not correspond to the trans-Golgi network (TGN). A 3D image collected of MDCK cells expressing GFP-MICAL-L1 showed that the vesicular distribution of the protein was clearly distinct from that of the furin, a marker of the TGN (Fig. 2A). We then investigate whether the vesicular structures associated with GFP-MICAL-L1 are endosomes, GFP-MICAL-L1 expressing cells were costained with several markers of endocytic pathways such as EEA1, a marker for early endosomes; Rab7, a late endosomal marker; and Rab11a, a recycling endosome marker. Other markers such as transferrin receptor (TfR), clathrin, Eps15, and Rab4a were also used (Fig. S2). MICAL-L1 localized to vesicle structures that show significant overlap with GFP-Rab7 (Fig. 2A). It showed a weak colocalization with Rab4a, Epsin and clathrin (Fig. S2), but was not detected in early endosomes or recycling endosomes involved in the slow recycling process as assessed by
colocalization with EEA1 and Rab11a respectively (Fig. 2A and S2). These data strongly suggest that MICAL-L1 resides in late endocytic compartments. We did not succeed to assess these features on the ultrastructural level as anti-MICAL-L1 or anti-GFP antibodies were not functional for electron microscopic analysis. To quantify the co-distribution of GFP-MICAL-L1 with Rab7, Rab4a, TfR, clathrin, Eps15 and EEA1, three-D image projections cross-correlation analysis was conducted on maximum Intensity projection (MIP) of three-D stacks of image as indicated in the method section. Consistent with our visual analysis, we found that the distribution of MICAL-L1 was significantly coincident with GFP-Rab7 and to a lesser extent with Rab4, clathrin and Eps15 (a marker of coated pits). The correlation of GFP-MICAL-L1 with EEA1 was very low suggesting a weak or no association of the protein with early endosomes (Fig. 2B).

MICAL-L1 protein contains a calponin domain thought to be important for interactions with actin filaments and microtubules (Suzuki et al., 2002; Terman et al., 2002). We examined the effect of Lat A or Nocodazole on the distribution of GFP-MICAL-L1. Lat A is a membrane-permeant actin monomer-sequestering drug capable of depolymerizing actin filaments [Sheff, 2002 #2547]. Treatment with Lat A disrupted stress fibers and redistributed actin to scattered dots in the cytoplasm. It also induced punctuate lateral actin structures. Treatment with Lat A for 30 min altered the localization of MICAL-L1 resulting in the redistribution of MICAL-L1 as cytoplasmic tubular structures. In contrast, depolymerization of microtubules with nocodazole did not affect the localization of MICAL-L1 (Fig. 3A). We then tested whether MICAL-L1 tubules formed after Lat A treatment were positive for Rab proteins. MICAL-L1 was detected as membrane tubules that overlapped extensively with Rab7, Rab8 and Rab13 (Fig. 3B).
MICAL-L1 regulates EGF receptor trafficking

In order to shed light on the function of MICAL-L1, we used a vector encoding short hairpin RNAs (shRNAs) to reduce MICAL-L1 expression in MDCK cells. The shRNA vector contains a GFP reporter gene which was used as a marker for transfected cells. Three different shRNAs targeting dog MICAL-L1 mRNA sequence and a control (scramble) sequence were expressed in stably transfected MDCK cells. We selected three clones corresponding to two different shRNA sequences that showed strong depletion of MICAL-L1 protein (Fig. 4A). Clone 2, MICAL-L1-shRNA (KD3) and clone 3, scramble (Scr1 as a control) were chosen for subsequent studies. We first investigated whether MICAL-L1 silencing affected the localization of cell-cell junction proteins. Knocking down MICAL-L1 expression did not significantly affect the localization of claudin1, occludin, ZO-1, and E-cadherin (Fig. 4B). 3D- images showed that in control and KD-cells, claudin1 and occludin were found at the lateral plasma membrane and as a punctuate staining in the cytoplasm. MICAL-L1 silencing did not alter Lamp1 (a late endosome-lysosome membrane protein) or Eps15 distribution (fig.4B, and S3).

Given the association of MICAL-L1 with late endosomes, we examine whether MICAL-L1 controls Transferrin (Tf) and EGFR endocytosis. To monitor the transport of Tf, MDCK cells on coverslips expressing GFP, GFP-MICAL-L1, Scr and MICAL-L1-shRNA were subjected to "pulse-chase" experiments with biotinylated-Tf. After the pulse, all cell lines analyzed exhibited similar distribution of Tf. After 30-min chase, GFP-MICAL-L1 expression or MICAL-L1 depletion did not alter Tf distribution. A cytoplasmic accumulation of Tf was observed (Fig. 5). We analyzed Tf distribution in two other independent Scr and KD clones which exhibited similar results (not shown). MICAL-L1 has been implicated in Tf receptor recycling in HeLa cells (Sharma et al., 2009). Our results were based on stable overexpression or depletion of MICAL-L1 in MDCK cells, in contrast to previous studies,
which were based on transient transfection in HeLa cells (Sharma et al., 2009). It is also possible that MICAL-L1 have additional functions in non epithelial cells.

We then investigated whether MICAL-L1 affected the trafficking of EGFR. Ligand binding to EGFR at the cell surface leads to rapid internalization of EGF-receptor complexes in early endosomes. Receptors are sorted at early endosomes, where, instead of being recycled, EGFR can be targeted to lysosomes for degradation. Receptors that are targeted to lysosomal degradation are distinguished from those destined for recycling by covalent addition of ubiquitin to the cytosolic domain of the EGFR (Sigismund et al., 2005; Huang et al., 2006; Roxrud et al., 2008; Sigismund et al., 2008).

Since ubiquitination is required for sorting/targeting EGFR to degradation, we then tested whether MICAL-L1 affects the EGFR ubiquitination. Cells were untreated or treated with EGF for 2 min at 37°C to stimulate maximum EGFR ubiquitination. EGFRs were immunoprecipitated using a monoclonal antibody against the extracellular domain of the receptor, separated by SDS-PAGE and immunoblotted with anti-Ubiquitin antibodies. Fig. 6A shows that in all cell lines analyzed, EGFR was ubiquitinated to similar extent, suggesting that ubiquitination of the EGFR was not affected by MICAL-L1. We also assessed the biochemical EGFR degradation in MDCK cells expressing Scr or MICAL-L1-shRNA. Indeed, cells depleted of MICAL-L1 (KD) exhibited a substantial EGFR degradation compared to control cells (Fig 6B-C). These results are consistent with the possibility that MICAL-L1 plays a role in EGFR trafficking at a late step of endocytic pathway.

In addition, immunofluorescence based "pulse-chase" assays with EGF were performed. After 15 min pulse, the distribution of endogenous EGFR was not affected by expression or depletion of MICAL-L1 as compared to control cells. The receptor was clearly detected as vesicular structures at perinuclear regions (Fig. 7). This perinuclear accumulation was also observed after 1h chase. After 3h chase, the EGFR was still accumulated at a perinuclear
endosomal compartment in control as well as in cells expressing GFP-MICAL-L1. In contrast, MICAL-L1-depleted cells exhibited different EGFR distribution. EGFR vesicles were not detected at the perinuclear region but were found in vesicles spread throughout the cytoplasm (Fig. 7). These results suggest that MICAL-L1 is required for EGFR trafficking at late stages of endocytosis. We also examined the influence of mock or MICAL-L1 depletion when a lower concentration of EGF was used (2 ng/ml). No significant differences between cells were observed (data not shown).

**Reintroduction of shRNA-resistant MICAL-L1 restores the peri-nuclear accumulation of EGFR in MICAL-L1-depleted cells**

We then checked that the effect of MICAL-L1-shRNA on EGFR trafficking was specifically caused by knockdown of MICAL-L1. For this purpose, we transfected cells depleted of MICAL-L1 with shRNA-resistant cherry-MICAL-L1 and performed a pulse-chase EGFR immunofluorescence experiments as indicated above. Cells were analyzed after 3h chase. Importantly, reexpression of cherry-MICAL-L1 in shRNA-treated cells restored the accumulation of EGFR at the perinuclear region of cells (Fig. 8A). We observed a marked accumulation of EGFR in cherry-MICAL-L1 positive late endosomes. The overlap of EGFR with endosomes that are Rab7 positive was less important. To check that Rab7 and MICAL-L1 positive late endosomes are dynamic, we assess their movement in living cells by using spinning confocal video-fluorescence microscopy. Movement of GFP-Rab7 and mcherry-MICAL-L1 was observed after addition of EGF to stimulate EGFR internalization (Fig. 8B and see supplementary material, Videos 1 to 3). Time-lapse video microscopy showed that GFP-Rab7 and cherry-MICAL-L1 were present within vesicles and tubules. These vesicles exhibited linear, bi-directional, saltatory motion in the cytoplasm. In this sequence, images were collected at 500 ms interval over a total time period of 12 min. Vesicles appeared to move rapidly in the cytoplasm with frequent pauses. They often encountered other tubulo-
vesicular structures. GFP-Rab7 and mcherry-MICAL-L1 were thus present in dynamic vesicular network, which corresponds to late endocytic compartments. Taken together, our data strongly suggest that at least a part of internalized EGFR is transported via MICAL-L1 and Rab7 endosomes. It also strengthens that MICAL-L1 is required for EGFR trafficking at late endosomal compartments.

**Rab13 colocalizes with MICAL-L1**

Since MICAL-L1 interacts with the GTP bound Rab13, we investigated whether Rab13 colocalized with MICAL-L1. Figure 9 shows that both proteins colocalized on tubulo-vesicular structures. Interestingly, we observed that mcherry-Rab13 exhibited a significant co-distribution with GFP-Rab7 and EGFR in non polarized epithelial cells (Fig. 9 and S4). The distribution of cherry-MICAL-L1 was also analyzed by immunofluorescence in MDCK cells expressing GFP-Rab13Q67L, GFP-Rab13WT or GFP-Rab13T22N (Fig. S5). In cells expressing GFP-Rab13 WT, and Q67L (GTP-bound form), cherry-MICAL-L1 decorated vesicle-like structures in the cytoplasm, MICAL-L1 was also detected at the cell periphery. In those cells, Cherry-MICAL-L1 colocalized with GFP-Rab13 WT and Q67L. In contrast, in cells expressing the inactive mutant GFP-Rab13T22N, cherry-MICAL-L1 was observed as tubulo-vesicular structures which did not colocalized with GDP-Rab13 mutant. Importantly, MICAL-L1 was not detected at the cell periphery (Fig. S5). These results are consistent with the interaction of MICAL-L1 with GTP-Rab13.

**Interaction of the N-terminal and C-terminal regions of MICAL-L1**

Given that MICAL-L1 has an inhibitory effect on EGFR trafficking, we hypothesized that MICAL-L1 exists as an inactive form due to an intramolecular interaction. First, we used the yeast two hybrid assay to test the interaction of MICAL-L1 with a truncated version containing CHL-PRD domains of MICAL-L1. We did not detect any interaction between these two protein constructs (data not shown). We then tested in vitro whether the NH2 – and
COOH-terminal regions of wild type MICAL-L1 interacted physically. We performed a GST pull-down assay using the C-terminal RBD fused to GST. The MICAL-L1 constructs used in the interaction studies were shown in Fig. 10A. GST alone or GST-RBD were immobilized on glutathione beads, and incubated with MDCK cell lysate derived from cell expressing GFP, GFP-CHL, GFP-PRD, and GFP-RBD. The proteins retained on the beads were separated on SDS-PAGE and blotted with an antibody against GFP. GST-RBD only retained the N-terminal CHL domain out of all the constructs examined while the GST control retained none (Fig. 10B). Thus the C-terminal RBD region of MICAL-L1 binds to the N-terminus. We next determined whether binding of Rab13 to the C-terminus of MICAL-L1 inhibits the interaction of RBD and CH. The CH domain was expressed as a His-CH fusion protein and incubated with GST-RBD beads. Fig. 10C confirms the direct interaction of RBD and CH domains and reveals that the CH domain is sufficient for binding to the C-terminus of MICAL-L1. Addition of increasing amounts of GTPγS-loaded His-Rab13 protein efficiently competed with binding of the CH to GST-RBD, thus indicating that CH/RBD interaction was controlled by GTP-Rab13. These data strengthen the idea that binding of Rab13 to MICAL-L1-RBD domain induces a conformational change in MICAL-L1 leading to its activation.

We then examined the effect of the two halves of MICAL-L1, CHLPRD and RBD, on EGFR degradation and on Rab13 localization. The CHLPRD domain contains putative SH3 interacting motives. The RBD domain binds Rab13, Rab7, Rab8a and Rab11a and has a NPF sequence at the N-terminus which is required for interaction with EHD proteins. Fig.S6 shows that RBD and CHLPRD exhibited a significant EGFR degradation compared to control cells. This suggests that both domains potentially interact with key elements involved in EGFR degradation. We found that GFP-RBD domain and mcherry-Rab13 colocalized and accumulated in the perinuclear region, whereas CHLPRD exhibited a partial colocalization with Rab13. Interestingly, CHLPRD and Rab13 were excluded from the perinuclear region.
and found as punctuate staining in the cytoplasm and at the cell periphery (Fig.S7). These results suggest that both domains are important for MICAL-L1 function. They are consistent with Rab13/RBD interaction.

Discussion

In this study, we found MICAL-L1, as a Rab13 interacting protein. Our data from the two hybrid screen, and the in vitro pulldown assay demonstrate that the interaction of Rab13 with MICAL-L1 is biologically relevant and GTP-nucleotide dependent. Like in MICAL, MICAL-L1-RBD domain shares significant homology with other coiled-coil domains in proteins of the Ezrin, Radixin, and Moesin (ERM) family that link plasma membrane to actin cytoskeleton (Terman et al., 2002). Moreover, MICAL-L1 contains a calponin homology (CH) and a LIM domain. LIM domains are conserved Zn$^{2+}$ finger motifs found in a variety of proteins exhibiting diverse biological roles. They act as modular protein binding interfaces mediating protein-protein interactions in the cytoplasm and the nucleus (Petit et al., 2003). The CH domain of MICAL-L1 shares high similarity to the CH domains identified in various actin associated proteins like α-actinin, spectrin, Vav and IQGAPs. CH domains either exist as tandem repeats of two CH domains or as a single CH domain. Depending on their context, they are thought to be important for interactions with actin, microtubules, and cytoskeleton-associated adaptor proteins (Gimona and Mital, 1998; Suzuki et al., 2002; Terman et al., 2002). The presence of a CH domain in MICAL-L1 emphasizes a potential role of MICAL-L1 in actin cytoskeleton. Interestingly, MICAL-L1 distribution is F-actin dependent. Although, we do not detect any interaction in vitro between actin and MICAL-L1 (A.Z., unpublished results), we cannot rule out the possibility that MICAL-L1 interacts with regulatory elements of the actin cytoskeleton. F-actin depolymerisation induces the membrane
tubulation, and tubules are associated with MICAL-L1, Rab7, Rab8a and Rab13. Therefore, we suggest that actin remodeling is important for the regulation of membrane transport mediated by MICAL-L1 and Rab7, Rab8a or Rab13 proteins. MICAL-L1-PRD region encompasses putative proline-rich, PxxP, sequences. It was reported that the proline-rich region of MICAL interacts with the SH3 domain of CasL, a protein required for β1 integrin-induced signal transduction and actin filament organization (Terman et al., 2002). Similarly, MICAL-L1 proline-rich motives could mediate interaction with SH3 domains of signaling proteins implicated in the regulation of membrane trafficking. Further investigations of MICAL-L1-PRD will be necessary to elucidate the binding partners and the signaling pathways mediated by the PRD domain.

Our data show that knocking down MICAL-L1 does not affect the distribution of cell-cell junction membrane proteins, suggesting that MICAL-L1 is not involved in cell-cell junction assembly. The PRD domain is not conserved in MICAL-L2, a protein that share similar domain organization with MICAL-L1 and interacts with Rab13 and Rab8a. Therefore, MICAL-L1 and MICAL-L2 may deserve different functions due to the presence of the PRD domain in MICAL-L1. MICAL-L2 regulates the recycling of tight junction membrane protein, occludin (Morimoto et al., 2005; Terai et al., 2006; Yamamura et al., 2008).

Neither silencing MICAL-L1 nor its overexpression affects EGFR ubiquitination. Importantly, silencing MICAL-L1 affects the distribution of the EGFR at a late stage of endocytosis, strengthening the notion that MICAL-L1 regulates ligand induced receptor endocytosis. Indeed, re-expression of MICAL-L1 in MICAL-L1-depleted cells leads to the accumulation of EGFR in MICAL-L1 positive late endocytic compartments; some of these compartments contain also Rab7. Moreover, the presence of MICAL-L1 in dynamic Rab7-positive vesicles confirms that MICAL-L1 regulates trafficking of the ubiquitinated EGFR at a late step of endocytosis. It is possible that MICAL-L1 is implicated in EGFR transport
between late endosomes and lysosomes since MICAL-L1 depletion stimulates EGFR degradation. It has been suggested that endosomes are organized as a mosaic of different Rab domains created through the recruitment of specific effector proteins which cooperatively act to organize membrane sub-domains (Sonnichsen et al., 2000; Rink et al., 2005). Therefore, MICAL-L1 by binding Rab7, Rab8a, Rab11a and Rab13, may help organize membrane domains required for cargo progression (EGFR, …) between late endocytic compartments. This is consistent with our finding that Rab13 colocalizes with Rab7 and EGFR at least in non-polarized cells. Further studies will be necessary to investigate the role of Rab13 in EGFR trafficking. Our results suggest that MICAL-L1 might have "open" and "closed" conformations. The two hybrid experiments (not shown) favors an intramolecular interaction of the N-terminal CH region with the C-terminal end (RBD domain), keeping MICAL-L1 in a closed inactive conformation. GTP-Rab13 disrupts this intra-molecular interaction in a dose dependent manner, suggesting that Rab13 binding to the RBD could induce a conformational change to convert MICAL-L1 to an opened form thereby unmasking potential sites required for the interaction of MICAL-L1 with other proteins. The interaction of MICAL-L1 with Rab13Q67L, but not Rab13T22N, and its localization as vesicles in GTP-Rab13 and as membrane tubules in GDP-Rab13 cells is in agreement with this hypothesis. This model for MICAL-L1 activation is also consistent with the fact that the RBD domain does not interact with MICAL-L1 full length (A.Z., unpublished results). A similar type of intra-molecular binding, controlled by Cdc42, Rho and Rab13 has been shown for N-WASP, mDia and MICAL-L2 proteins respectively (Sakane et al., 2010; Watanabe et al., 1999; Rohatgi et al., 2000). The binding of Rab13 to MICAL-L2 promotes the recruitment of α-actinin4 to the tips of neurites. Similarly, we propose that Rab13 binding to MICAL-L1 controls MICAL-L1 intra-molecular interaction to facilitate the interaction with proteins involved in the regulation
of different steps of late endocytic pathways. Further investigations will be necessary to identify MICAL-L1 partners.

In conclusion, MICAL-L1, a multidomain adaptor protein could serve as a platform for the regulation of a subset of transmembrane proteins sorting/targeting at late endocytic compartments.

**Materials and Methods**

**Antibodies**

The purified GST-RBD protein (aa 690-863 of MICAL-L1) was injected into rabbits in order to generate polyclonal antibodies. The resulting antiserum was consecutively affinity purified on a GST column and a RBD column. Monoclonal antibodies against GFP were purchased from Boehringer Mannheim, (Indianapolis, USA) respectively. Anti-E-cadherin antibodies were purchased from Transduction Laboratories (San Diego, CA), anti-claudin1 and anti transferrin receptor from Zymed (Zymed Laboratories Inc., San Francisco, CA), rat monoclonal anti ZO-1 antibody R40.76 was used as previously described (Anderson *et al.*, 1988). Mouse monoclonal anti-EGFR was purchased from Calbiochem (Darmstadt, Germany). Polyclonal anti-EGFR and anti-Ub were from Santa Cruz Biotechnology (CA, U.S.A.), polyclonal anti-caveolin from Transduction laboratories (Lexington, KY) and polyclonal anti-Rab13(product N°, HPA003996) from Sigma (St Louis, USA) and anti-Eps15 from santa cruz Biotechnology

**Two-hybrid screening and isolation of full-length cDNA**

The yeast reporter strain L40 (Mata) was transformed with pVJL12-Rab13Q67L by electroporation. Several transformants were checked by immunoblot in order to determine the expression levels of the fusion protein. The clone with the highest expression level was chosen for the two-hybrid screen. A library derived from human placenta was cloned into
pGAD1318 followed by transformation into the strain Y187 (Matα). 10x10⁹ transformants were conjugated to approximately 100x10⁹ L40 cells by 8 hours mass mating in liquid culture. The mating was controlled by placing aliquots under a light microscope in order to determine the percentage of diploids. After 8 hours the cells were plated on synthetic medium lacking leucine, tryptophan and histidine. Finally 35 positive clones were isolated and inserts were sequenced, followed by comparison to the human genome. Plasmids containing sequences of interest were rescued into *Escherichia coli* HB101 cells plated on leucine-free media. Plasmids derived from positive clones were newly transformed into Y187 cells and another mating assay was performed. Diploids that could grow in selective medium were finally tested for β-galactosidase activity. Out of the 35 positive clones, 18 contained overlapping fragments of 600 to 850bp length. The fragments were sequenced and translated in frame to the N-terminal DNA-binding domain of Lex A used for the two-hybrid screen. Thereby, a partial sequence encoding a putative open reading frame could be detected. This sequence was named Rab13 binding domain (RBD). By sequence comparison, several EST cDNAs could be identified that contained the RBD sequence. The longest EST containing an insert of about 5800bp length was purchased from the IMAGE consortium (IMAGE clone 2262785) and sequenced. Sequence analysis revealed an ORF of 2586 bp encoding a protein of 863 amino acids.

**Constructs**
GFP-Rab7 is from M. Arpin, mcherry-Rab11a from B. Goud and GFP-Rab4a from P. Chavrier (Curie Institute Paris). GFP-Rab8a was generated (Marzesco et al., 2002). For overexpression experiments, a 3000bp fragment encoding the full length MICAL-L1 was cut out of IMAGE clone 2262785 by a XhoI-XbaI digest and ligated into the corresponding sites of a pEGFP-N2 vector (Clontech, Pako Alto, CA) thereby replacing the cut-out GFP. This plasmid now contained the MICAL-L1 ORF, 68bp of the 5’-untranslated region as well as
200bp of the 3’-untranslated region. MICAL-L1 wild type was fused to the C-terminus of the enhanced green fluorescent protein (GFP) and cloned into the pGFP-C3 vector (Clontech Inc. Palo Alto, CA). Cherry-MICAL-L1 was also generated. All constructions were verified by sequencing.

For RNAi, Canine MICAL-L1 cDNA sequence (XM_538381) was used to design three pairs of primers targeting different regions of canine MICAL-L1 open reading frame according to the manufacturer instructions (InvivoGen, San Diego, CA 92121). The shRNA sequences that efficiently inhibited MICAL-L1 expression were: shRNA oligo sens1, 5’-ACCTCGTGCG CAGTATTACAACCACTTTCAAGAGAAGTGTTGTAATCT-GCGACTT-3’; shRNA oligo-antisens1, 5’-

_CAAAAAGTCGCAGTATTACAACCACTTCTCTTGAAAGTGTTGTA

ATACTGCAGCGG-3’. shRNA oligo sens2: 5’

ACCTCGACCTACGTGTCCGAGTATTATCAAGAGTAATACGTACACGCTAGGTTT
-3’. shRNA oligo-antisens2, 5’-

_CAAAAAGACCTACGTGTCCGAGTATTACTCTCTTGATAATACGTACACGCTAGGTC

G-3’

Scrambles oligos sens and antisens were: 5’-ACCTCGCTGTTCC

TACTCGAAAAATCAAGAGTTATTTGCAGTAGGAAACAGCTT-3’, and 5’-AAAA

_AAGCTGTTTCTACTCGAAAAATAACTCTCTTGATTATTTCGAGTAGGAACAGCG-3’

respectively. Italics indicate the 7 base pairs hairpin loop. Underlined nucleotides correspond to Bbs1 cloning sites

**Cell culture and Transfection**

Mardin-Darby canine kidney (MDCK) cells (clone II) were cultured in DMEM supplemented with 10% fetal calf serum, 2mM glutamine, 100 U/ml penicillin and 10 mg/ml streptomycin. The cells were incubated at 37°C under 10% CO₂ atmosphere. Cells were grown for 3 days;
under these conditions, cells were subconfluentes and not fully polarized. MDCK cells stably expressing GFP, GFP-Rab13T22N or GFP-Rab13Q67L were previously described (Marzesco et al., 2002). Stable MDCK cell lines expressing MICAL-L1 wild type (FL) was generated by transfection using the lipofectamine 2000 transfection reagent according to the manufacturer instructions (InvivoGen, San Diego, CA 92121). Positive clones were selected and cloned in the same medium supplemented with 1 mg/ml of G418 (Life Technologies, Inc.). Stable transfected clones were maintained under selection in 500 μg/ml of G418.

For RNAi transfection, the sens and antisense oligonucleotides were annealed and cloned into Bbs1 sites of the psiRNA-h7SK vector (InvivoGen, San Diego, CA 92121). This vector contains Zeocin resistance and encodes the GFP reporter gene used as a marker for transfected cells. The psiRNA-h7SK-scramble vector was used as a negative control. The shRNA constructs were transfected into MDCK II cells using the lipofectamine 2000 transfection reagent. Stable MDCK cells expressing shRNA of MICAL-L1 and scramble (control) were selected and cloned in the same medium supplemented with 0.3 mg/ml of Zeocin. Stable transfected clones were maintained under selection in 0.1 mg/ml of Zeocin.

**GST pull down assay**

The GST fusion protein was constructed by inserting a BamHI-XhoI fragment containing RBD into the corresponding sites of pGEX-4T expression vector. GST-fusion proteins were expressed in *E.coli* and purified according to the manufacturer’s protocol (Amersham Pharmacia, Uppsala, Sweden). Purified GST-RBD or GST alone was bound to glutathione beads (Amersham Pharmacia, Uppsala, Sweden) and incubated with cell extracts derived from MDCK cells expressing GFP-Rab13Q67L and GFP-Rab13T22N or GFP and GFP-MICAL-L1. The beads were washed three times with PBS and bound material was analyzed on SDS-PAGE and immunoblotted using monoclonal anti-GFP or anti-Rab13 antibodies.
The cDNAs encoding Rab13 and CH domain (amino acids, 1-102) were inserted into *E. coli* pET-15b expression vector, produced as histidine fusion proteins (His-Rab13 and His-CH), and purified on Ni^{2+}-agarose beads by classical methods.

**Immunoblot and Coimmunoprecipitation**

Protein amounts were determined using the Bradford protocol (Bio-Rad, Munich, Germany), and protein separation in SDS-PAGE and immunoblotting performed as described (Kohler et al., 2004). Immunoblot detection was done by ECL according to manufacturer protocols (GE, Healthcare).

For immunoprecipitation experiments, MDCK cell lines expressing GFP-MICAL-L1 or MICAL-L1 kD were solubilized in IP-buffer (50 mM Tris-HCl pH 7.6, 120 mM NaCl, 1.8 mM CaCl2, 0.5% Triton X-100, protease inhibitor mix). Cell extracts were centrifuged at 15000g and the supernatant incubated for 4 hours at 4°C with anti-MICAL-L1 or anti-EGFR antibodies followed by the addition of protein A-sepharose. After 2 hours incubation, the beads were washed five times with IP buffer. Bound material was solubilized with SDS-PAGE sample buffer and analyzed by immunoblotting with anti-MICAL-L1, anti-GFP or anti Ubiquitin antibodies.

**Immunofluorescence Microscopy**

Cells grown on glass coverslips for 3 days were washed with PBS containing 1 mM calcium and 0.5 mM magnesium and fixed with 3% paraformaldehyde for 15 minutes. Immunofluorescence was performed essentially as described (Marzesco et al., 2002). For permeabilization, cells were incubated in blocking buffer (PBS+0.2% BSA containing 0.1% TritonX-100). Permeabilized cells were incubated for 1 hour with the primary antibodies, rinsed three times for 10 min with the blocking buffer and then incubated with affinity purified secondary antibodies raised in goat and conjugated to Cy2 or Cy3 (Jackson Immunoresearch Laboratories, West Grove, PA). After washing, samples were analyzed with
a 3-D microscope (see next section) and further processed with Adobe Photoshop Software (Adobe Systems, Mountain View, CA).

**Image acquisition, processing and fluorescence quantification**

3D stacks with a 0.3 μm step were acquired by three-dimensional deconvolution microscopy (Angenieux *et al.*, 2005), adapted on an Eclpise 90i upright microscope (Nikon, S.A., France) equipped with a cool SNAP HQ2 CCD camera (Photometrics, Tucson, USA) and using a 100x CFI Plan Apo VC objective NA 1.4 (Nikon, S.A., France) controlled in the Z axis by a Piezo Objective (PI, S.A.S, France). All deconvolution processes were performed automatically using an iterative and measured PSFs based algorithm (Gold-Meinel) on batches of image stacks, as a service proposed by the PICT-IBiSA imaging facility of the Curie Institute. The levels of colocalization were estimated on deconvolved images by Intensity Correlation Analysis (ICA), basically as previously described (Li *et al.*, 2004) and using the dedicated ImageJ plugin (Correlation Analysis Sasha), except that we did not select particular area of the images through image segmentation in this case. Quantitative analyses of coincident structures and ICA based on their relative intensities in the different channels were performed on Maximum intensity Projections (M.I.P.) of the multiple labeled stacks constituted of an average of 8 to 15 planes each.

**Video Microscopy**

Images were acquired on a fast scanning confocal system (with CSU22 Yokogawa spinning-disk head) mounted on an inverted microscope Nikon TE2000 equipped for live cell imaging (the Box, LIS, SW). Image acquisitions were performed using a 100× Plan Apo VC 1.4 oil objective and a highly sensitive cooled interlined charge-coupled device (CCD) camera (Roper CoolSnap HQ2). Z-dimension positioning was accomplished by a Piezo motororized stage (NanoScanZ – Piezo focusing stage, Marzhauser). Both 491nm and 561nm laser diodes interfaced with an AOTF (Accousto Optic Tunable Filter) were used for sample illumination.

**Transferrin uptake**
Cells were grown on coverslips and serum starved for 1h. They were incubated with 25 μg/ml of biotinylated holo-transferrin (sigma T-3915) for 30 min at 0°C. After washing, cells were incubated for 15 min-pulse at 37°C and chased in normal DMEM medium for 30min at 37°C. Cells were processed for immunofluorescence and biotinylated transferrin was revealed by streptavidin-texas red.

**EGFR endocytosis**

Cells expressing the indicated plasmids or transfected with shRNA of MICAL-L1 were serum starved for 16h, pretreated with cycloheximide (CHX, 40 μg/ml) at 37°C for 60 min, and then cooled to 4°C. Subsequently, 100 ng/ml EGF was applied at 4°C in the presence of CHX to allow ligand binding to the receptor. Cells were then pulsed for 15 min at 37°C, washed and chased in low serum medium plus CHX for the indicated time. To answer visualization of internalized EGFR, coverslips were washed and stripped for 3 minutes at 4°C in 50 mM glycine, 100mM NaCl, pH 3.0 to remove surface labelling and then subjected to immunofluorescence analysis.

**EGFR ubiquitination**

For ubiquitination, cells were serum starved for 4 hours and untreated or treated with EGF (100ng/ml) at 37°C for 2 min. Cells were washed with phosphate buffered saline (PBS) and solubilized at 4°C in 150 mM KCl, 2 mM MgCl₂, 20 mM HEPES, 10% glycerol, pH 7.2, 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 544 μM iodoacetamide, 10 μg/ml aprotinin containing 1% TritonX-100 and 10mMN-ethyl-maleimide (NEM)(Huang et al., 2006). Lysates were cleared by centrifugation at 14000g for 15 min.
EGFR was immunoprecipitated with monoclonal antibody, separated by SDS-PAGE and immunoblotted with anti-ubiquitin antibodies.

**EGFR degradation assay**

Cells were serum starved for 4h in the presence of 40 μg/ml CHX, and stimulated with 100 ng/ml of EGF in the presence of CHX for 15 min at 37°C. Cells were washed and chased in low serum medium plus CHX for the indicated time. They were lysed in 10 mM tris-HCl pH 7.6, 150 mM NaCl, 25mM KCl, 1.8 mM CaCl2, 1% Triton X-100, and a mixture of protease inhibitor. Cells extracts were then cleared by centrifugation, separated by SDS-PAGE and immunoblotted with polyclonal anti-EGFR antibodies. Protein bands were quantified using image J software (NIH)

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**References**


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Figure legends

Figure 1. MICAL-L1 interacts with the GTP-, but not with the GDP-bound Rab13. (A) Two hybrid interaction of Rab13 binding domain (RBD) with Rab13. The yeast reporter strain L40 (Matα) transformed with pVJL12-RBD was conjugated by patch mating to strain Y187 (Matα) expressing cDNAs of small GTPases (indicated on top) cloned into pGAD1318. Diploid cells were plated on synthetic medium lacking leucine, tryptophan and histidine and were grown at 30°C. Growth phenotype was determined after 48 hours. RBD interacts only with the WT and the active form (Q67L) of Rab13, but not with the inactive form (T22N) of Rab13 or other small GTPases. (B) GST alone or a GST-RBD fusion protein was purified from *E. coli*, bound to glutathione beads and incubated with cell extracts derived from MDCK cells expressing GFP-Rab13Q67L and GFP-Rab13T22N. Bound proteins were analyzed by immunoblotting with a monoclonal anti-GFP antibody. Given that GFP-Rab13Q67L was highly expressed than GFP-Rab13T22N, only 50% of GFP-Rab13Q67L lysate were incubated with GST-RBD beads. (C) GST-RBD fusion protein bound to glutathione beads and incubated with cell extracts derived from MDCK cells expressing GFP, GFP-Rab4, GFP-Rab7, GFP-Rab8 and GFP-Rab11. Bound proteins were analyzed by immunoblotting with a monoclonal anti-GFP antibody (D) MICAL-L1 protein expression in mammalian cell lines and placenta. 25 μg of total extracts from NIH3T3, CHO, Cos, CaCo2, LLC-PK1, MDCK cells and placenta extract were analyzed by immunoblotting with the affinity purified anti-RBD antibodies. MICAL-L1 was detected as a band of 116K.
Figure 2. **MICAL-L1 is associated with late endocytic compartments.** Coverslips of cells expressing GFP-MICAL-L1 or cherry-MICAL-L1 were processed for immunofluorescence using (A) anti-furin, anti-EEA1, cherry-Rab11a and GFP-Rab7. 3D-projections of images were collected. The projections were combined into a single color image in the third column (merge). Colocalization of the two fluorophores (green and red) was revealed by the yellow
color resulting from their overlapping emissions. MICAL-L1 significantly colocalizes with Rab7. The distribution of GFP-MICAL-L1 with the four endocytic markers was emphasized in the 2.0 x insets. Bar, 10 \( \mu \text{m} \). (B) Intensity correlation analysis, comparing the distribution of MICAL-L1 with either Rab7, Rab4a, TfR, clathrin, Eps15 and EEA1 from 3D-projection images of cells. The correlation coefficients of double labels were then calculated on deconvolved images with Intensity Correlation Analysis as indicated in the method section. The distribution of MICAL-L1 is significantly correlated with late endocytic compartments associated with Rab7. I. C.A was performed on full image fields of reconstituted Maximum Intensity projection of 3D stacks of at least 10 planes. At least 5 and up to 10 double labeled cells were analyzed in each field. Averaged quantification as shown in bar graphs was done at least on 3 different experiments.
**Figure 3.** MICAL-L1 distribution depends on Filamentous actin (F-actin). (A) MDCK cells expressing GFP-MICAL-L1 were treated with either Latrunculin A (LatA) or nocodazole (Noc) and processed for immunofluorescence with phaloidin or with anti-α-tubulin. 3D stacks were acquired by three-dimensional deconvolution microscopy. 3D-projections of images are shown. (B) MDCK cells co-expressing mcherry-MICAL-L1 and either GFP-Rab7, GFP-Rab8a or GFP-Rab13 were treated with LatA and processed for immunofluorescence. 3D stacks were acquired by three-dimensional deconvolution microscopy. 3D-projections of images are shown. Bar 10 μm.
Figure 4. Knocking down MICAL-L1 does not affect the distribution cell-cell junction proteins and lamp1. Epithelial MDCK cells were stably transfected with shRNA targeted to dog MICAL-L1 mRNA sequence indicated as KD or mock transfected indicated as scramble (Scr). KD1 and 2 correspond to shRNA sequence 1, KD3 to shRNA sequence 2 (A) Cell lysates from the 3 different stable clones expressing either Scr or KD were immunoblotted with anti MICAL-L1 antibodies. α-tubulin expression was also detected as a loading control. (B) MDCK cells expressing Scr1 or MICAL-L1 KD3 were grown on coverslips for 3 days; under these conditions, cells were not fully polarized. Cells were processed for immunofluorescence with claudin1, occludin, ZO-1, E-cadherin, and Lamp1 antibodies. Note that knocking down MICAL-L1 does not perturb the distribution of Lamp1 and cell-cell junction proteins. Three independent stable MDCK clones KD and three Scr clones were tested for the distribution of cell-cell junction and lamp1 proteins. They all gave similar results. 3D-projections of images are shown. Bar, 10 μm.
Figure 5. MICAL-L1 does not affect transferrin (Tf) distribution. Cells on coverslips were grown for 3 days, serum starved for 1h, washed and incubated with 25 μg/ml of biotinylated holo-transferrin for 30 min at 0°C. After washing, cells were pulsed for 15min, and then chased in DMEM medium for 30 min. Coverslips were processed for immunofluorescence. 3D stacks were acquired by three-dimensional deconvolution microscopy. 3D-projections of images are shown. Bar, 10 μm
Figure 6. Effect of MICAL-L1 on EGFR ubiquitination and degradation (A) cells were untreated or treated with EGF for 2 min at 37°C. EGFR was immunoprecipitated using a monoclonal antibody against the extracellular domain of the receptor. Equal amount of proteins were separated by SDS-PAGE and immunoblotted with anti-Ubiquitin antibodies. Tubulin was detected as a loading control. (B), Cells were serum starved for 4h in the presence of 40 μg/ml cyclohexamide (CHX), and stimulated with 100 ng/ml of EGF for 15 min in the presence of CHX. They were washed and chased for the indicated time points. They were lysed and endogenous EGFR was analyzed by immunobloting using a polyclonal anti-EGFR antibodies. (C), quantification of three independent experiments performed as in B. After blotting, EGFR bands were quantified using image J program (NIH image, Rockville, MD) and Microsoft Excel software.
Figure 7. MICAL-L1 affects EGFR distribution. Stables MDCK cells expressing GFP, Scr1 (as control), MICAL-L1 shRNA (KD3), GFP-MICAL-L1 were serum starved for 16 h, pretreated with CHX and incubated with 100 ng/ml EGF in the presence of CHX. Cells were then pulsed for 15 min at 37°C, washed and chased in low serum medium plus CHX for the indicated time points. They were washed and stripped with acidic buffer to remove surface labeling. Cells were then analyzed by immunofluorescence and 3D stacks were acquired by three-dimensional deconvolution microscopy. 3D-projections of images are shown. EGFR staining was mainly detected at a perinuclear region after 15 min EGF pulse, or after 1h chase. After 3h chase, MICAL-L1-depleted cells exhibited a different EGFR distribution. The receptor was distributed in vesicles spread throughout the cytoplasm. Bar, 10 μm
Figure 8. Overexpression of cherry-MICAL-L1 leads to EGFR accumulation at late endosomes. (A) MICAL-L1-depleted cells were co-transfected with shRNA-resistant cherry-MICAL-L1 and GFP-Rab7, subjected to EGF stimulation for 15 min and chased for 3h. Cells were fixed and stained with anti-EGFR (blue). 3D stacks were acquired by three-dimensional microscopy and 3D-projections of images are shown. Triple colocalization is indicated in white. Inset shows magnified example of colocalization. EGFR largely accumulated in cherry-MICAL-L1 positive endosomes. A partial overlap of EGFR and GFP-Rab7-positive and cherry-MICAL-L1 late endosomes was noticed. Bar, 10 μm. (B) MDCK cells transiently cotransfected with GFP-Rab7 and mcherry-MICAL-L1 were stimulated with EGF as in A. Cells were imaged by video spinning confocal microscopy at 37°C. Images were collected at 500ms intervals (25 ms exposure per channel). A sequence of 8 images (from 0 to 10 min 40 s) indicates the movement of a GFP-Rab7 and mcherry-MICAL-L1 positive late endosome. Arrows denote GFP-Rab7 and mcherry-MICAL-L1 vesicle. Bar, 2 μm. A movie containing these images (0-40min) highlights an example of GFP-Rab7 and mcherry-MICAL-L1 positive late endosomes behavior is shown (supplementary information, movies 1 to 3).
Figure 9. Rab13 colocalizes with MICAL-L1 and Rab7. MDCK cells co-expressing GFP-Rab13/mcherry-MICAL-L1 or cherry-Rab13/GFP-Rab7 were processed for immunofluorescence. 3D stacks were acquired by three-dimensional deconvolution microscopy. 3D-projections of images are shown. Bar, 10 μm
Figure 10. Intra-molecular association of CH with RBD domain of MICAL-L1. (A), Schematic representation of GFP-MICAL-L1 constructs. Three GFP-tagged mutants, CHL (CH+LIM), PRD, RBD and GFP-tagged wild type (FL) constructs were generated. Numbers represent corresponding amino acids  (B) GST alone or GST-RBD were immobilized on
glutathione beads, and incubated with lysates of MDCK expressing various GFP-tagged fragments of MICAL-L1. The proteins retained on the beads were separated on SDS-PAGE and blotted with an antibody against GFP-tag. (C) Recombinant His-Rab13 preloaded with GTPγS was mixed at various concentrations with GST-RBD beads and His-CH. Proteins bound to the beads were separated by SDS-PAGE and blotted with the anti-His antibodies. Addition of increasing amounts of GTPγS-loaded His-Rab13 protein efficiently competes with binding of the CH to GST-RBD. The left panel shows purified His-Rab13 and His-CH proteins. The Rab13 lower band (15k) recognized by anti-His antibodies results from degradation of His-Rab13.
Supplemental material

Figure S1. Amino acid sequence and domain organization of MICAL-L1. (A) Deduced amino acid sequence of MICAL-L1. The calponin homology (CH) domain (aa 7-107), the LIM motif (aa 188-196), the RBD (aa 690-863), and putative PxxP sequences in the PRD (proline rich region: aa 216-680) are underlined. The RBD contains 2 putative leucine-zipper motives ranging from aa 715 to 739. (B) MICAL-like 1 and 2 have a similar domain organization. Amino acid identity between human MICAL-L1 and 2 is indicated within arrows. CH, calponin homology domain, PRD, proline-rich domain is not conserved in MICAL-L2, RBD, Rab13 binding domain, and aa, amino acid.

Figure S2. Cells expressing GFP-MICAL-L1 were processed for immunofluorescence with anti-transferrin receptor (TfR), anti-clathrin, anti-Eps15 or transfected with GFP-Rab4a. 3D-projections of images were collected. The projections are combined into a single color image in the third column (merge). The 2.5 x insets emphasize the distribution of MICAL-L1 and different endocytic markers. Bar, 10 μm

Figure S3. Cells expressing GFP-MICAL-L1 and shRNA-MICAL-L1 (KD3) were processed for immunofluorescence with anti-Eps15. 3D-projections of images were collected. Note that knocking down MICAL-L1 does not alter Eps15 distribution. Bar, 10 μm

Figure S4. MDCK cells expressing GFP-Rab13 were treated with EGF for 15 min plus CHX, washed and chased for 3 h in low serum medium. They were analyzed by immunofluorescence for GFP-Rab13 and EGFR. 3D-projections of images were collected. Bar, 10 μm

Figure S5. MDCK cells expressing GFP-Rab13WT, GFP-Rab13Q67L or GFP-Rab13T22N were transfected with cherry-MICAL-L1 and processed for immunofluorescence. 3D stacks were acquired by three-dimensional deconvolution microscopy. 3D-projections of images are shown. Rab13WT and Rab13 GTP (Q67L) colocalize with MICAL-L1. Bar, 10 μm
**Figure S6.** (A) Cells expressing GFP-CHLPRD (CHL+PRD) and RBD domains were serum starved for 4h in the presence of 40 μg/ml cyclohexamide (CHX), and stimulated with 100 ng/ml of EGF as in Fig.6. They were lysed and endogenous EGFR was analyzed by immunobloting using a polyclonal anti-EGFR antibodies. (B), quantification of three independent experiments performed as in A. After blotting, EGFR bands were quantified using image J program (NIH image, Rockville, MD) and Microsoft Excel software.

**Figure S7.** Cells expressing GFP-CHLPRD or GFP-RBD domains and mcherry-Rab13 were processed for immunofluorescence. 3D-projections of images were collected. Note the accumulation of GFP-RBD and mcherry-Rab13 at the perinuclear region. Bar, 10 μm
Legend to movie 1, 2 and 3

MDCK cells were cotransfected with GFP-Rab7, and mcherry-MICAL-L1. Then, cells were treated with EGF (100 ng/ml) for 15 min in the presence of cycloheximide. Cells were washed and chased as in figure 6. Images were acquired on a fast scanning confocal system (with CSU22 Yokogawa spinning-disk head) mounted on an inverted microscope Nikon TE2000 equipped for live cell imaging, temperature (37°C) and atmosphere 5% CO₂, (the Box, LIS, SW). Image acquisitions were performed using a 100× Plan Apo VC 1.4 oil objective and a highly sensitive cooled interlined charge-coupled device (CCD) camera (Roper CoolSnap HQ2). Z-dimension positioning was accomplished by a Piezo motororized stage (NanoScanZ–Piezo focusing stage, Marzhauser). Both 491nm and 561nm laser diodes interfaced with an AOTF (Accousto Optic Tunable Filter) were used for sample illumination. Frames were captured over a 5 min time period, and converted into Quicktime movies.