Regulation of Src trafficking and activation by the endocytic regulatory proteins MICAL-L1 and EHD1

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MICAL-L1 controls Src activity

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Abstract

Localization of the non-receptor tyrosine kinase c-Src (Src) to the cell periphery is required for its activation and to mediate focal adhesion turnover, cell spreading and migration. Inactive Src localizes to a perinuclear compartment and endocytic transport mediates Src movement to the plasma membrane. However, the precise pathways and regulatory proteins responsible for Src transport are incompletely understood. Here we demonstrate that Src partially co-localizes with the endocytic regulatory protein Molecule Interacting with CasL-Like1 (MICAL-L1) in mammalian cells. Furthermore, MICAL-L1 is required for growth factor and integrin-induced Src activation and transport to the cell periphery in HeLa cells and human fibroblasts. Accordingly, MICAL-L1 depletion impairs focal adhesion turnover, cell spreading and cell migration. Interestingly, we find that the MICAL-L1 interaction partner Eps15 Homology Domain-containing protein 1 (EHD1) is also required for Src activation and transport. Moreover, EHD1 recruitment to Src-containing recycling endosomes by MICAL-L1 is required for Src release from the perinuclear endocytic recycling compartment in response to growth factor stimulation. Our study sheds new light on the mode by which Src is transported to the plasma membrane and activated, and provides a new function for MICAL-L1 and EHD1 in the regulation of intracellular non-receptor tyrosine kinases.
Introduction

Cell adhesion and migration are fundamental processes that contribute to normal physiologic functions such as organ development, tissue maintenance and homeostasis. These processes are also intricately involved in pathologic conditions such as wound healing, atherosclerosis and cancer (Friedl & Gilmour, 2009). The non-receptor tyrosine kinase c-Src (Src), the founding member of the Src Family Kinases, plays key roles in regulating cell adhesion and migration (Fincham & Frame, 1998), and deregulation of Src kinase activity in cancer is correlated with metastasis and poor survival (Wheeler et al, 2009). Each SFK is characterized by a prototypical structure defined by a: N-terminus lipid modification (Koegl et al, 1994), SH4 unique domain, type II proline-rich binding SH3 domain, phospho-tyrosine binding SH2 domain (Payne et al, 1993), proline-rich linker region, SH1 kinase domain containing a critical autophosphorylation site (Smart et al, 1981), and a C-terminal regulatory domain (Cooper et al, 1986) containing a critical tyrosine residue (Y529 in human Src) that is phosphorylated by C-Terminal Src Kinase (Nada et al, 1991).

The domain architecture of Src is central to regulating its kinase activity (Xu et al, 1999). Phosphorylation of Y529 promotes an intramolecular interaction with its SH2 domain, which in turn brings the proline-rich SH2-kinase linker domain in close proximity to the SH3 domain thus preventing Src kinase activity. Activation of receptor tyrosine kinases such as EGFR and PDGFR (Kypta et al, 1990; Luttrell et al, 1994; Mori et al, 1993), or engagement of integrin receptors with the extracellular matrix (Arias-Salgado et al, 2003), promotes protein-protein
interactions with the SH3 domain or the SH2 domain, or dephosphorylation of Y529 (Ren et al, 2004). This in turn alleviates the Src intramolecular interaction and facilitates kinase activity. Once activated, Src phosphorylates multiple proteins that promote: changes in membrane dynamics through modulation of Rho GTPase activity (Timpson et al, 2001), focal adhesion disassembly (Fincham & Frame, 1998), and actin cytoskeletal rearrangement (Chang et al, 1995; Tanji et al, 2010), all of which collectively contribute to cell migration.

While biochemical regulation of Src is one mode of regulating its kinase activity, it is now apparent that modulation of Src intracellular localization is an important regulatory mechanism (Sandilands & Frame, 2008). Initial studies demonstrated that v-Src (constitutively active Src that is the oncogenic component of the Rous Sarcoma Virus) localizes to the plasma membrane (PM) and cytoplasmic compartments (Courtneidge et al, 1980; Rohrschneider, 1979). Temperature sensitive mutants of v-Src that are inactive at 41°C and active at 35°C demonstrated that inactive v-Src localizes to the perinuclear region, and upon activation, translocates to peripheral membranes and focal adhesions (Welham & Wyke, 1988) to promote focal adhesion turnover and migration (Fincham & Frame, 1998).

Similarly, inactive Src localizes to perinuclear endosomes whereas active Src localizes to focal adhesions (Kaplan et al, 1994; Kaplan et al, 1992). Elegant work by Sandilands et al. using wild-type Src expressed in Src-null mouse embryonic fibroblasts provided clear evidence that Src translocates to the cell periphery in an actin-dependent manner within endocytic vesicles containing the
small GTPase RhoB. Disruption of the actin cytoskeleton by cytochalasin D or
genetic ablation of RhoB prevented Src translocation and activation (Sandilands
et al, 2004). Data from other groups suggest that trafficking of Src occurs
through late endosomes (Kasahara et al, 2007), regulated by the ESCRT
complex (Tu et al, 2010). However, the effect of ESCRT-depletion on Src activity
may be indirect, as the ESCRT complex regulates the trafficking and localization
of integrins in human fibroblasts (Lobert & Stenmark, 2012). Regardless, the
endocytic regulatory proteins that are involved in Src localization remain largely
uncharacterized.

Work from our lab and others has shown that Eps15 homology (EH)
domain-containing protein-1 (EHD1) and its interacting partner Molecule
Interacting with CasL Ligand Like-1 (MICAL-L1) are required for the exit of
transmembrane receptors from the perinuclear endocytic recycling compartment
(ERC) (Caplan et al, 2002; Sharma et al, 2009). While MICAL-L1 and EHD1 are
crucial mediators of receptor recycling to the PM, the potential role of MICAL-L1
and EHD1 in regulating the intracellular trafficking and function of non-receptor
tyrosine kinases such as Src has not been established. We hypothesized that
MICAL-L1 and EHD1 may be required for Src transport to the cell periphery and
thus may be regulators of Src-dependent functions. We demonstrate herein that
Src localizes to MICAL-L1-positive endocytic compartments. Furthermore,
MICAL-L1 and EHD1 depletion impairs Src activation and localization to the
periphery following growth factor treatment. Functionally, we show that the loss
of MICAL-L1 impairs focal adhesion turnover, cell spreading and cell migration.
We now demonstrate that inactive Src is concentrated in the perinuclear ERC, and that upon growth factor treatment, EHD1 is recruited to MICAL-L1-positive structures and promotes the release of Src from the ERC allowing Src to travel to the PM, thereby affecting key downstream cellular processes.

Results

**MICAL-L1 associates with Src in mammalian cells and is required for Src activation and localization to the cell periphery.**

In HeLa cells, MICAL-L1 displays a highly unique localization, decorating long tubular endosomes that emanate from the ERC ((Sharma et al, 2009) and Fig. 1B). Under steady-state conditions, we observed that endogenous Src partially co-localized with MICAL-L1 along tubular membranes that radiate from the ERC (Figure 1 A and B; arrows in inset). Given that Src family members such as Fyn and Yes may also be expressed in HeLa cells, we next over-expressed Src-GFP and HA-MICAL-L1 fusion proteins in Src/Yes/Fyn (SYF) null mouse embryonic fibroblasts to test if MICAL-L1 specifically associates with Src in mammalian cells. At steady-state conditions, Src-GFP is concentrated along membrane ruffles and tubulo-vesicular structures underneath the PM (Fig. 1D; arrows). HA-MICAL-L1 is also found associated with these structures (Fig. 1E; arrows) and partially co-localizes with Src-GFP (Fig. 1F; merge), suggesting that MICAL-L1 specifically associates with Src and not other Src family members such as Yes and Fyn. Although a Src and MICAL-L1 could be weakly observed in a complex (unpublished observations), it is likely that the insolubility of
membrane-associated Src renders this interaction minimal (Fra et al, 1994). Moreover, the failure to detect an interaction by yeast two-hybrid experiments suggests that the interactions are indirect. These data support the notion of a transient MICAL-L1-Src interaction, consistent with interactions described between Src and other proteins involved in regulating its transport (Sandilands et al, 2004; Tu et al, 2010).

Given that Src localizes to MICAL-L1-decorated endosomal structures, we hypothesized that MICAL-L1 may regulate Src activation and transport. Src autophosphorylation on tyrosine 419 (in humans) is required for kinase activity (Feder & Bishop, 1990; Reuter et al, 1990) and serves as a surrogate marker for Src activation. In control-siRNA treated HeLa cells, stimulation with EGF caused a time-dependent increase in Src activation (Fig. 2A, lanes 1-3). However, in MICAL-L1-siRNA treated cells, >95% of the MICAL-L1 was depleted and Src-pY419 levels failed to increase above the baseline observed in serum-starved cells (Fig. 2A, lanes 4-6). This indicates that MICAL-L1 is required for EGF-induced Src activation. In agreement with these findings, MICAL-L1-depletion inhibited localization of active Src (green) to paxillin-positive peripheral focal adhesions (red) (Fig. 2, compare B to C; see line-scan analysis).

While we hypothesized that MICAL-L1 knockdown (KD) directly impacts Src localization and activation, it was necessary to rule out the possibility that MICAL-L1 might indirectly impair EGF-induced Src activation by affecting EGFR surface levels or endocytosis. Using flow cytometry with fluorescently labeled EGF, we found that MICAL-L1-depleted cells displayed a small increase in EGF
binding capacity compared to control cells (Fig. S1 A, B), indicating that lack of EGFR on the plasma membrane is not the underlying cause of reduced Src activation upon MICAL-L1 KD. To evaluate whether MICAL-L1 KD affects EGFR internalization, cells were pulsed with unlabeled EGF for 15 minutes and immunostained with antibodies against EGFR. MICAL-L1 KD cells displayed no overt EGFR trafficking defects (Fig. S1 C-F). Lastly, MICAL-L1-depletion did not affect EGFR phosphorylation upon stimulation with EGF (Fig. S1 G; compare levels of EGFR-pY1068). Of note, Src does regulate EGFR trafficking (Donepudi & Resh, 2008; Ware et al, 1997; Wilde et al, 1999). Thus, MICAL-L1 KD may have subtle effects on Src-dependent EGFR trafficking. Given that MICAL-L1 KD has minimal effects on EGF binding and EGFR activation, we concluded that decreased Src activation in MICAL-L1 KD cells is not due to indirect effects.

The failure of active Src to localize to the cell periphery upon MICAL-L1-depletion raised the question of its subcellular distribution under these conditions. To address this, we used an antibody that recognizes both the phosphorylated and non-phosphorylated forms of Src, and monitored Src localization by immunofluorescence microscopy in the presence and absence of MICAL-L1. In serum-starved cells either in the presence (Fig. 2D) or absence (Fig. 2F) of MICAL-L1, Src was observed throughout the cell (including the PM), but displayed a concentrated presence in the perinuclear region (arrows). In control-siRNA cells, stimulation with EGF caused Src to disperse from the perinuclear region and display enhanced localization underneath the PM (Fig. 2E). However, in MICAL-L1-depleted cells, upon EGF-stimulation, the majority of Src remained
in the perinuclear region (Fig. 2G; arrows), indicating that MICAL-L1 is required for Src transport from this region to the PM.

Given the contradictory reports as to whether inactive Src localizes to the ERC (Sandilands et al, 2004) or late endosomes (Kasahara et al, 2007; Tu et al, 2010), we addressed the nature of the perinuclear region in which Src is retained upon MICAL-L1-depletion. We stimulated cells with EGF and immunostained for Src and transferrin receptor (TfR), a well-established marker of the ERC that is absent in late endosomes. Compared to EGF-stimulated control cells, which display modest overlap between Src and TfR (Fig. 2H), MICAL-L1-depletion resulted in substantial accumulation and overlap of both Src and TfR in the ERC (Fig. 2I; yellow arrows). Quantification of Src fluorescence in the TfR-positive ERC indicated that it was significantly higher in MICAL-L1 KD cells (Fig. 2J).

Accordingly, MICAL-L1 co-localizes with endogenous Src in HeLa cells (Fig. 1) and regulates EGF-induced Src activation by controlling the exit of Src from the ERC (Fig. 2).

**MICAL-L1 co-localizes with Src and is required for its recruitment to circular dorsal ruffles in human foreskin fibroblasts following PDGF stimulation**

While MICAL-L1 partially co-localizes with Src and regulates its endocytic trafficking in HeLa cells upon EGF stimulation (Fig. 1 and 2), it was unclear whether MICAL-L1 co-localizes with Src in other cells such as normal human fibroblasts and whether the regulation of Src by MICAL-L1 is specific to EGF or
constitutes part of a more general regulatory mechanism. In Src null mouse embryonic fibroblasts, transfected Src translocates from the ERC to the PM ruffles upon platelet derived growth factor (PDGF) stimulation (Sandilands et al, 2004). However, the localization of endogenous Src in human fibroblasts following PDGF stimulation has not been characterized. In human foreskin fibroblasts (BJ cells), Src localized to circular dorsal ruffles following PDGF stimulation (CDR; compare Fig. 3 A to D, see arrow). Similarly, PDGF stimulation resulted in MICAL-L1 recruitment to CDR, where it co-localized with Src on tubulo-vesicular structures (Fig. 3 E and F; arrow, see insets), suggesting that the MICAL-L1/Src co-localization is physiologically relevant in normal human cells. MICAL-L1 also co-localized with other focal adhesion regulatory proteins such as phosphorylated focal adhesion kinase (FAK) (Figure 3G-I; arrow and arrowhead) and phosphorylated paxillin (Figure 3J-L; arrows).

Given the co-localization of MICAL-L1 and Src along CDR and that Src activity is required for CDR formation and macropinocytosis (Mettlen et al, 2006; Veracini et al, 2006), we tested if MICAL-L1 is required for Src localization to CDR in BJ cells. Control and MICAL-L1-depleted BJ cells were stimulated with PDGF for 10 minutes and stained with Src and phalloidin (to mark actin). In control cells, Src localized to uniformly round actin-rich positive CDR (Fig. 4 A). In contrast, MICAL-L1 KD resulted in a significant reduction of Src recruitment to CDR (Fig. 4B; quantified in Fig. 4C), and those structures were frequently elliptical rather than circular (Fig. 4B). In addition, the size of MICAL-L1 KD cells was typically greater than that of control cells (compare Fig. 4B to A; compare
Fig. 5D-F to A-C and see cell spreading experiments in Fig. 7A-D). To assess if MICAL-L1 KD affected CDR maturation into macropinocytic vesicles, we quantified the number of cells containing enlarged Rab5 and Rabankyrin-5 vesicles (all measured at greater than 1 μm in diameter), two well-established markers of macropinosomes (Mettlen et al, 2006; Schnatwinkel et al, 2004). Compared to control cells, in which 46% of cells contained macropinosomes (Fig. 4 D-F; yellow arrows, quantified in J) after 20 minutes of PDGF stimulation, only 10% of MICAL-L1 KD cells had macropinosomes (Fig. 4 G-I; quantified in J).

Given that Src activation at the periphery is required for CDR formation in mouse fibroblasts (Azimifar et al, 2012), it is interesting that CDR form in MICAL-L1 KD cells at similar rates to control cells. However, we found that MICAL-L1 KD CDR displayed altered morphology by live-cell imaging (unpublished observations) and MICAL-L1 KD clearly affected CDR maturation into macropinosomes (Fig. 4D-I), thus MICAL-L1 may also directly affect CDR maturation in addition to its function in trafficking Src to PDGF-induced CDR in human fibroblasts.

**MICAL-L1 is required for PDGF-induced focal adhesion turnover**

Recent studies have demonstrated that integrins and other focal adhesion proteins (such as paxillin and FAK, Fig. 3) rapidly redistribute to CDRs upon PDGF stimulation and that this redistribution is linked to focal adhesion turnover and migration (Gu et al, 2011; Hoon et al, 2012). We first addressed whether MICAL-L1 KD in human fibroblasts affected focal adhesions at steady-state. As depicted, MICAL-L1 was easily depleted in these cells with a range of different
concentrations of SiRNA (Fig. S2A). A modest reduction in pY419-Src was observed in MICAL-L1 KD cells, with little or no impact on pY397-FAK, pY118-paxillin or actin (Fig. S2A). MICAL-L1-depleted BJ cells displayed larger, more prominent focal adhesions under steady-state conditions (Fig. S2, compare E to D for vinculin and G to F for paxillin). Furthermore, active Src was largely absent from focal adhesions in MICAL-L1-depleted cells (Fig. S2, compare C to B), suggesting that focal adhesion dynamics may be impaired due to lack of active Src recruitment. Because Src kinase activity is required for focal adhesion turnover (Fincham & Frame, 1998), we hypothesized that loss of MICAL-L1 may impair PDGF-induced focal adhesion turnover in BJ cells.

Following PDGF stimulation, Src and the focal adhesion protein vinculin co-localized along CDR in control cells (Fig. 5 A-C; arrows), in agreement with our findings and those from other labs that focal adhesion proteins redistribute to CDRs. However, MICAL-L1-depletion caused considerable Src retention in the perinuclear region (Fig. 5E; arrowheads) and impaired vinculin re-distribution to CDRs (Fig. 5D). Control and MICAL-L1 KD cells both form actin-positive CDR (Fig. S3 B,E, yellow arrows), thus the failure of vinculin to localize to CDR (Fig. S3 A,D) is not because CDR do not form in MICAL-L1 KD cells. MICAL-L1-depleted cells displayed increased vinculin-positive focal adhesions (Figure 5D). To test if MICAL-L1-depletion impairs focal adhesion turnover, which would explain the increased number of focal adhesions, we quantified the number of focal adhesions in PDGF-stimulated cells. As shown in Figure 5G-I and quantified in Figure 5M, control-siRNA-treated cells displayed a significant time-
dependent decrease in the number of vinculin-containing focal adhesions (serum-starved=24.6 ± 4.1; 15 min. PDGF=14.5 ± 3.1). Under non-stimulated conditions, MICAL-L1-depletion caused a significant increase in focal adhesions/cell (serum-starved=56.2 ± 8.7) relative to control (Fig. 5J and M). Moreover, stimulation of MICAL-L1-depleted cells did not lead to focal adhesion disassembly (PDGF=55.1 ± 8.8). Reintroduction of MICAL-L1 into MICAL-L1-depleted cells partially rescued focal adhesion number (SS=24.3 ± 3.3, PDGF=28.4 ± 0.71; quantified in Figure 5M with representative images in Fig. S3 G-J). Furthermore, reintroduction of MICAL-L1 to MICAL-L1 KD cells partially rescued PDGF-induced cellular elongation, a phenotypic marker we used to assess cell migration in fixed cells (Fig. S4).

To further address the impact of MICAL-L1-depletion on increased focal adhesion stability, we quantified focal adhesion size, as this is linked to impaired turnover (Webb et al, 2004). Focal adhesion size in BJ cells was categorized into three pools: small (1-5 μm²), medium (6-10 μm²) and large (11-30 μm²). Figure 6 A-D is a representative example of fields of cells that have been quantified, with the following corresponding figures: 6A=5G, 6B=5J, 6C=5I, 6D=5L. PDGF stimulation of control BJ cells (Fig. 6C) caused the disassembly of larger focal adhesions that are present under serum-starved conditions (Fig. 6A). The statistics of focal adhesion size distribution are depicted in Figure 6E. The majority of focal adhesions in control cells are between 1-5 μm² (serum starved=93.5%, PDGF 97.5%). In contrast, MICAL-L1-depletion led to a
decrease in the small focal adhesion population (88.1%) that does not change substantially following PDGF stimulation (89.8%).

While there are many Src substrates present at focal adhesions, FAK phosphorylation at position Y925 is crucial for focal adhesion turnover (Deramaudt et al, 2011). If Src activity is decreased at focal adhesions in MICAL-L1-depleted cells, we hypothesized FAK-Y925 phosphorylation would be impaired. BJ cells were stimulated with PDGF and immunoblots were used to measure FAK-Y925 phosphorylation. In control cells, there was a sharp increase in Y925 phosphorylation following 5 and 15 min. PDGF stimulation (Fig. 6F Lane 1-4). This rise in phosphorylation coincided with increased levels of active Src. However, MICAL-L1-depleted cells displayed impaired PDGF-induced activation of Src (Lane 5-8). Consequently, no increase in FAK-Y925 phosphorylation was observed. These data reinforce the notion that MICAL-L1 regulates the transport of active Src to focal adhesions, thus controlling focal adhesion turnover.

**MICAL-L1 regulates cell spreading**

Adhesion molecules such as integrin receptors mediate cell attachment to the extracellular matrix. Upon attachment, cell spreading requires the continual formation and disassembly of focal adhesions and Src is crucial for this process (Huveneers & Danen, 2009). To test if MICAL-L1 is required for cell spreading in BJ cells, control siRNA-treated or MICAL-L1-siRNA-treated cells were plated on fibronectin-coated coverslips for 90 min. Control cells spread and polarized
displaying long actin stress fibers with vinculin positive focal adhesions localized to the end of actin stress fibers at the cell periphery (Fig. 7A).

In contrast, MICAL-L1-depleted cells failed to polarize on fibronectin-coated coverslips (Fig. 7B-D). We observed several abnormal phenotypes in these cells characterized by: large round cells with prominent actin cables distributed throughout the cell (Fig. 7B; arrows), polygonal cells with disorganized actin stress fibers (Fig. 7C), or cells with a single broad lamellipodia containing actin arcs (Fig. 7D; arrowhead). MICAL-L1-depleted cells were also larger (3537 µm² ± 754) compared to control cells (2098 µm² ± 455) and contained more focal adhesions per cell (MICAL-L1-siRNA= 49.2 ± 10.7, compared to control= 21.6 ± 6.6; see Fig. 7E and F).

Having established that MICAL-L1 is required for proper BJ cell spreading, we tested if integrin-induced Src activation was affected by MICAL-L1 depletion. When control treated BJ cells were plated onto fibronectin, Src activation increased compared to cells held in suspension (Fig. 7G, lanes 1-4). MICAL-L1-deficiency caused a modest decrease in Src activation upon fibronectin plating (Lanes 5-8; arrow), as observed by decreased levels and the delayed appearance of FAK-Y925 (Fig. 7G, lanes 5-8) compared to control cells. Reduced Src activation and Src-dependent FAK phosphorylation is consistent with the impaired focal adhesion turnover we observed during cell spreading (Fig. 7 B-D).

**MICAL-L1-depletion affects directional cell migration**
Since focal adhesion turnover and spreading are both necessary for cell migration, we hypothesized that MICAL-L1 might be a regulator of cell migration. To test this idea, we used scratch wound assays in control cells and in MICAL-L1-depleted cells. BJ cells migrated into the wound within 24 h (Fig. 8 A-C). In comparison, MICAL-L1-depleted cells were impaired in wound closure (Fig. 8 D-F). Src and MICAL-L1 co-localize along the leading edge (Fig. 8G and H; arrowhead) and along tubules at the cell front (Fig. 8G and H; inset and arrow) of migrating cells. Since Src is required for cell polarization to the wound (Magdalena et al, 2003; Timpson et al, 2001), we assessed polarity in migrating fibroblasts using the orientation of the Golgi apparatus and the actin cytoskeleton as well established markers of polarization. While the majority of control cells displayed Golgi oriented to the wound and stress fibers that were perpendicular to the wound (Fig. 8J; 68%), MICAL-L1-depleted cells were partially impaired in their ability to polarize to the wound (Fig. 8K; 40%).

Cell migration requires formation and disassembly of focal adhesions at the cell front and focal adhesion disassembly at the cell rear (Wozniak et al, 2004). In control cells, there were increased numbers of focal adhesions as well as increased intensity of vinculin staining at the cell front compared to the dimmer, less frequent staining at the cell rear (Fig. 8L). In comparison, MICAL-L1-depleted cells displayed an overall increase in the number of focal adhesions (in agreement with our previous data) but failed to polarize in the direction of the wound (Fig. 8M). Taken together, these results demonstrate the requirement of MICAL-L1 for polarized cell migration in BJ cells.
EHD1 is required for Src transport and activation and acts as a molecular pinchase on MICAL-L1 tubules to release Src from the ERC in response to EGF

Having established that MICAL-L1 co-localizes with Src and regulates its trafficking and activation in response to a variety of stimuli, we sought to understand the mechanism by which MICAL-L1 regulates Src. In the absence of a direct interaction between MICAL-L1 and Src, we postulated that MICAL-L1 regulates Src release from the ERC and its subsequent activation by recruiting vesiculating proteins such as EHD1. This hypothesis is further strengthened by the fact that we have previously shown that EHD1 also regulated focal adhesion turnover and cell migration in fibroblasts (Jovic et al, 2007); however, the mechanism by which this occurs remains unclear. We hypothesized that impaired Src recruitment to focal adhesions could explain these deficits. Indeed, EHD1 KD in HeLa cells led to a significant retention of Src in the TfR positive ERC (Fig. 9 A-F; see arrows; quantified in G). In addition, EHD1 KD impaired EGF-induced Src activation (Fig. 9H; compare control lanes 1-3 to EHD1 KD lanes 4-6).

We have recently shown that MICAL-L1 is a regulator of recycling tubule biogenesis in HeLa cells (Giridharan et al, 2013). Interestingly, MICAL-L1 may also facilitate the cleavage or vesiculation of recycling tubules by recruiting molecular pinchases such as EHD1 (Cai et al, 2013). Whether or not MICAL-L1 and EHD1 constitutively bind to one another in cells, or if EHD1 is recruited to
MICAL-L1 by certain stimuli remains unclear. Therefore, we tested if EGF stimulation in HeLa cells affected EHD1 or MICAL-L1 sub-cellular localization. Under steady-state conditions, EHD1 concentrated in the perinuclear region (Fig. 10A) but was also found on a subset of MICAL-L1 tubules. MICAL-L1 also concentrated in the perinuclear region; however, MICAL-L1 tubules emanating from the ERC were also visible (Fig. 10B) with many being EHD1-negative. Serum-starvation resulted in the diffuse displacement of EHD1 from the ERC (Fig. 10D) and a modest increase in MICAL-L1 tubulation and spreading of the tubular endosomes out of the ERC (Fig. 10E). Surprisingly, EGF stimulation had dramatic effects on MICAL-L1-decorated tubular recycling endosomes and EHD1 localization. First, MICAL-L1 tubules were substantially shortened and displaced to the cell periphery (Fig. 10H; see arrows). Secondly, in contrast to serum-starved cells, EGF induced an apparent increase in concentrated EHD1 to MICAL-L1 tubular structures (Fig. 10G; arrows) suggesting that EGF may either enhance the recruitment of EHD1 to tubules or possibly stimulate EHD1 ATPase activity leading to the vesiculation of MICAL-L1-containing tubular recycling endosomes.

While the molecular mechanisms governing increased recruitment of EHD1 to MICAL-L1 tubules in response to EGF are unclear, the fact that EGF induced dramatic changes to MICAL-L1 recycling tubules was unexpected and suggested that the release of Src from the ERC in response to EGF was may be mediated by the recruitment of EHD1. Therefore, we tested if EGF-induced vesiculation of MICAL-L1 tubules was dependent on EHD1. Similar to our
previous experiments, EGF stimulation led to dramatic shortening, peripheral scattering, and reduction of MICAL-L1 tubules compared to serum starved in control cells (Compare Fig. 10 K to J; quantified in N). In contrast, EHD1 KD resulted in a modest increase of MICAL-L1 tubular area under serum-starved conditions (Fig. 10 L) that did not decrease with EGF stimulation (Fig. 10 M; quantified in N). In summary, we conclude that MICAL-L1 and EHD1 regulate Src trafficking and activation. MICAL-L1 recruits EHD1 to perinuclear ERC endosomes allowing for the release of Src from ERC and transportation to the PM, where Src functions in mediating actin cytoskeletal rearrangement (CDR), focal adhesion disassembly, and cell migration (Fig. 11; see model).

Discussion

Cell adhesion and migration requires the correct spatiotemporal activation of many kinases, phosphatases, and GTPases, with Src kinase being central to the entire process (Huveneers & Danen, 2009). Insufficient Src activation paralyzes the cell and prevents turnover of focal adhesions (Fincham & Frame, 1998) while excessive Src activation can promote cancer cell spreading, invasion and metastasis (Wheeler et al, 2009). Accordingly, exquisite regulation of Src activity is paramount to normal cell behavior.

We found that Src localizes to MICAL-L1-positive tubular recycling endosomes in HeLa cells (Fig. 1) and that MICAL-L1 and its binding partner EHD1 are required for stimulation-induced Src transport and activation (Fig. 2 and 9). HeLa cells have an extensive network of recycling tubules, potentially
due to differential expression of individual EHD proteins and/or a slow rate of ATP hydrolysis and fission, and are thus a useful tool for studying recycling. In other cells, including fibroblasts, the morphology of the ERC is less compact, more dispersed, and harder to visualize. However, we also investigated the localization of Src and MICAL-L1 in human fibroblasts (Fig. 3). Although some MICAL-L1 and Src can be detected on tubular recycling endosomes in these cells, we were surprised to find that in addition to small, presumably lipid-rich tubular structures (not shown), MICAL-L1 localized to PDGF-stimulated, actin-rich CDR where it co-localized with Src. Since normal fibroblasts are an excellent model to study CDR and focal adhesions, we used the two cell lines intermittently to study the regulation of Src by MICAL-L1. Overall, the fact that MICAL-L1 co-localized with Src in human fibroblasts and was crucial for Src recruitment and activation (Fig. 4 and 6) was consistent with our data in HeLa cells, allowing us to generalize that regulation of Src localization and activity by MICAL-L1 is general and not specific to one cell type.

While our data provide new information regarding the regulation of Src transport out of the ERC (Fig.11 ERC-PM pathway), it does not address the other arm of the Src endocytic regulatory pathway (Fig.11 PM-ERC pathway). How a stimulus provided at the cell surface (such as EGF) is able to promote the movement and activation of an intracellular protein localized to the ERC remains unknown. Sandilands and Frame proposed a novel mechanism in which a small pool of Src localizes to the PM, even in the absence of stimulus (Sandilands & Frame, 2008). Upon growth factor stimulation or integrin activation, this small
pool of PM Src is activated, whereby it translocates to the ERC (Fig. 11; blue dashed arrow). This would initiate a positive-feedback loop to activate the larger pool of ERC-localized Src. One phenomenon that we commonly observed was that MICAL-L1 and EHD1-depleted cells had relatively more active Src under serum-starved conditions compared to serum-starved control cells and MICAL-L1- or EHD1 KD-stimulated cells (refer to immunoblots in Figures 2, 6, 7, and 9). This lends support to the idea that the loss of MICAL-L1 or EHD1 may trap a relatively larger pool of Src at the PM under serum-starved conditions (compared to control cells).

Accordingly, it is possible MICAL-L1 and EHD1 also play a role in in the PM-ERC pathway (Fig. 11), as MICAL-L1 also regulates the transport of some receptors into the perinuclear ERC by its interaction with the Collapsin Response Mediator Protein-2 (CRMP2) and dynein, which modulate microtubule-based transport (Rahajeng et al, 2010). Interestingly, the localization of Src to the ERC is microtubule-dependent (Fincham et al, 2000). Indeed, nocodazole-induced disruption of microtubules stabilizes focal adhesions, whereas nocodazole washout promotes synchronous disassembly of focal adhesions (Ezraty et al, 2005). We speculate that the re-establishment of the microtubule network promotes Src transport into the ERC, activation of Src, and its subsequent transport to the PM for disassembly of focal adhesions. Future studies will be needed to dissect the specific contribution of microtubules and microtubule motor proteins to Src activation and how this regulates microtubule-induced focal adhesion turnover.
Lastly, we found that MICAL-L1 recycling tubules (in HeLa cells) are highly dynamic. Specifically, EGF stimulation induced a rapid shortening of MICAL-L1 tubules (Fig. 10). The molecular mechanisms regulating this phenomenon remain enigmatic, however, our data clearly implicates EHD1 in mediating EGF-induced MICAL-L1 vesiculation. While EHD1 and other EHD family members are phosphorylated in response to serum (Fichtman et al, 2008), it is unclear what role phosphorylation or other potential post-translational modifications have on EHD ATPase activity or endocytic membrane recruitment.

Our data clearly demonstrate the importance of MICAL-L1 and EHD1 in regulating Src ERC-PM transport and activation. The finding that MICAL-L1-depletion affects Src-dependent processes such as focal adhesion dynamics, adhesion and migration underscores the \textit{in vivo} relevance of the Src-MICAL-L1-containing tubular endosomes, and highlights the potential significance of MICAL-L1 and EHD1 in regulating non-receptor kinases.
Materials and Methods

Reagents and Antibodies

Recombinant human PDGF-BB, EGF and EGF-Rhodamine were purchased from Invitrogen. Fibronectin was purchased from Sigma. The following antibodies were used: EHD1 (Caplan et al, 2002), vinculin (Sigma), GM130 (BD Biosciences), Src (36D10), phospho-Src (tyrosine 416; D49G4), FAK, phospho-FAK (tyrosine 925), EGFR, phospho-FAK (tyrosine 1068):Cell Signaling Technologies, phospho-FAK (tyrosine 397) and phospho-paxillin (tyrosine 118): Invitrogen, actin and MICAL-L1: Novus, phospho-Src (tyrosine 416; immunofluorescence): Millipore, human transferrin receptor (Zymed).

Cell Culture

Cervical cancer cell line HeLa cells (ATCC-CCL2) and SYF mouse embryonic fibroblasts (ATCC-CRL2459; (Klinghoffer et al, 1999)) were grown in DMEM (high glucose) containing 10% fetal bovine serum, penicillin/streptomycin (Invitrogen) and 2 mM glutamine. Normal human foreskin fibroblasts (BJ; ATCC-2522) were grown in EMEM containing 10% fetal bovine serum, penicillin/streptomycin, 2 mM glutamine and non-essential amino acids.

Plasmids, siRNA Transfections and Rescue Experiments

Human c-Src-GFP was created similarly to that described previously (Sandilands et al, 2004). Briefly, human Src (Invitrogen- IOH12563) was PCR amplified using forward primer 5’ ccgctcgagatggtagtaacaagagcaagcc 3’ and reverse primer 5’
cccaagctttgatctgatccggaggtctccccgggtcgg 3’. The resulting PCR product, which contains (from 5’ to 3’): 5’ Xho1 restriction site, c-Src ORF, gly-ser-gly-ser linker and a 3’ HindIII site were ligated into pre-digested EGFP-N1 (Clontech) and verified by sequencing. HA-MICAL-L1 was previously described (Sharma et al, 2009). Plasmids were transfected into SYF cells using FuGene HD (Roche).

Pooled and individual oligonucleotides targeting human MICAL-L1, custom EHD1 siRNA (Sharma et al, 2009) and non-targeting control siRNA were obtained from Dharmacon. HeLa cells were transfected with Oligofectamine (Invitrogen) with 150 nM siRNA while BJ cells were transfected using Lipofectamine RNAiMAX (Invitrogen) with 50 nM siRNA. Efficiency of protein knockdown was measured 48-72 h post-transfection by immunoblot or immunofluorescence for each experiment. For rescue experiments, 48 h post siRNA transfection 8 X 10^5 BJ cells were electroporated using Amaxa Nucleofector II according to the manufacture’s protocol with a siRNA-resistant GFP-MICAL-L1 construct.

_Growth factor stimulation and phenotype analysis_

Cells grown on glass coverslips (HeLa) or 10 μg/ml fibronectin-coated cover-slips (BJ) were serum starved overnight (18 h) in Opti-MEM media. Cells were then stimulated with EGF (50 ng/ml) or PDGF (15 ng/ml) for the times indicated prior to immunoblotting or immunofluorescence.

Src ERC fluorescence was measured using ImageJ. Cells were stimulated with EGF, fixed and stained with Src and TfR antibodies (as
Images were imported into ImageJ and a region of interest was created in individual cells by manually tracing the perinuclear TfR to mark the ERC region. Src fluorescence was quantified using the ‘measure’ function. All cells (control and KD cells in individual experiments) were fixed, stained and imaged on the same day under identical confocal settings.

Src recruitment to PDGF-induced CDR (15 CDR/condition/experiment, three independent experiments total) was quantified from Src and actin fluorescence values measured by profile line-scan analysis in Pascal LSM5 Image Examiner. Data was expressed as a ratio of Src fluorescence to actin fluorescence. The total MICAL-L1-containing tubule area was calculated as described previously (Cai et al, 2013).

**Immunoblotting**

Cell lysates were prepared by washing cells two times in ice-cold PBS. Cells were then scraped off the plate with a rubber policeman into ice-cold RIPA buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 1% Triton, 0.5% sodium deoxycholate, 0.1% SDS, 1.8 mg/ml iodoacetamide, 1 mM orthovanadate, 2.5 mM sodium pyrophosphate, 1 mM glycerophosphate, 1 μg/ml leupeptin, 5 μg/ml aprotinin, 1 mM PMSF). Lysates were then clarified by centrifugation at 13,000 RPM at 4°C. Protein levels were quantified using the BCA assay (Biorad). For immunoblotting, 20-30 μg (HeLa cells) or 10-15 μg (BJ cells) of protein lysate/sample was separated by 8% SDS-PAGE. Proteins were transferred onto nitrocellulose membranes. Membranes were blocked for 1 h at room temperature in TBST
(TBS +0.1% Tween). 3% BSA was used for blotting with phospho-protein antibodies or TBST+5% dry milk and then incubated overnight in primary antibodies diluted in either TBST-BSA (for phospho-proteins) or TBST-milk. Membranes were washed with TBST and then incubated with HRP-conjugated goat anti-mouse (Jackson Research Laboratories) or donkey anti-rabbit (GE Healthcare) secondary antibody for 1 h at room temperature.

**Cell Spreading**

72 h post-siRNA transfection, BJ cells were detached from plates with 0.05% trypsin/EDTA. Trypsin was inactivated by addition of complete growth medium. Cells were pelleted and washed twice in serum-free media and then incubated in suspension at 37°C for 30 min. Cells were then plated onto 10 μg/ml fibronectin-coated coverslips for 90 min. For immunoblots, cells in suspension were plated onto fibronectin-coated tissue culture dishes and harvested at the time points indicated in text. Cell area was measured using Pascal LSM image examiner by manually tracing borders around cells. Focal adhesions were quantified as described below.

**Focal adhesion quantification**

Focal adhesion number and size was measured in ImageJ. Images from vinculin stained samples were imported into ImageJ. Cropped images of single cells were assessed with a common threshold. The total number of focal adhesions per cell was quantified using ‘measure particles’ function with parameters set to
measure particles 1-30 μm². Focal adhesion size distribution was analyzed by categorizing focal adhesion area into three categories: 1-5 μm², 6-10 μm², and 11-30 μm².

**Scratch wound assay**

48 h post-siRNA transfection, BJ cells were trypsinized and plated onto 10 μg/ml fibronectin-coated cover-slips at high density in low serum (2%) media overnight. A single scratch was made using a P200 pipet tip. Cell debris was washed away with low serum media and cells were then incubated in low serum media for the times indicated in text prior to fixation.

**Flow Cytometry**

48 h post-siRNA transfection, control and MICAL-L1 KD cells were trypsinized. Trypsin was inactivated by addition of complete serum. Cells were pelleted and washed twice with serum free media. Cells were incubated in suspension in serum free media for 1 h at 37°C. Cells were then pelleted at 4°C and resuspended in ice-cold serum free media containing 1 μg/ml EGF-Rhodamine and incubated at 4°C with gentle rotation. Cells were pelleted, washed three times with cold PBS and fixed with 4% paraformaldehyde. At least 10,00 cells for analyzed for surface bound EGF-Rhodamine by flow cytometry analysis (BD Biosciences).

**Immunofluorescence**
Cells were treated as indicated in the text and then fixed in 3.7% paraformaldehyde in PBS (HeLa) or PHEM (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl₂, pH 7.0) for 15 min. at room temperature. Cells were rinsed three times in PBS and permeabilized in 0.1% Triton-X/PBS for 3 min. (HeLa) or 10 min. (BJ). Cells were then blocked for 1 h at room temperature in PBS containing 10% normal goat serum, 1% BSA and 0.1 M glycine. The cells were incubated with primary antibody in PBS/1% BSA, and where indicated, phalloidin-AlexaFluor 488 or Phalloidin-Rhodamine (Invitrogen), for 1 h at room temperature, washed 3X in PBS and then incubated with appropriate fluorochrome-conjugated secondary antibodies (Molecular Probes) plus DAPI for 1 h at room temperature. Cells were washed 3X in PBS and mounted in Fluoromount G.

Single plane confocal images were collected using Zeiss LSM5 Pascal laser confocal microscope with a Plan-Apochromat 63X/1.4 oil objective except for scratch wound images which were imaged using Plan-Neofluor 10x/0.3 objective. For quantification, collected images were imported into ImageJ or LSM Pascal Image Examiner as described above. Images presented in figures were imported into Adobe Photoshop CS, where they were re-sized and formatted to 300 dpi resolution with minimal image manipulation (whole-image adjustment of brightness was done using ‘levels’ function).

Statistics
Data from ImageJ was imported into Microsoft Excel. Mean and standard error of the mean were calculated from data obtained from three independent experiments. Statistical significance was calculated by One-way ANOVA and Tukey test (when comparing more than two samples) using the Vassarstats program (www.vassarstats.net).
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Author Contribution

JBR carried out the majority of the experiments, prepared the figures and wrote the manuscript. DK assisted in carrying out a number of the experiments and prepared key reagents required for several figures in the manuscript. NN and SC jointly directed the study, assisted in figure preparation, manuscript preparation and editing.

Conflict of Interest

The authors have no conflict of interest to declare.

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**Figure Legends**

**Figure 1** Partial co-localization between MICAL-L1 and Src in mammalian cells.  
(A-C) Immunofluorescence demonstrating partial co-localization of endogenous Src (A) along endogenous MICAL-L1-decorated tubular endosomes (B) in HeLa cells as marked by arrows in inset.  
(D-F) Over-expressed Src-GFP (D) partially co-localizes with HA-MICAL-L1 (E) in SYF fibroblasts (arrows).  
Blue=DAPI, scale bar=10 μm.

**Figure 2** MICAL-L1-depletion in HeLa cells impairs EGF-induced Src activation and translocation out of the ERC.  
(A) Immunoblot of control (lanes 1-3) and MICAL-L1-depleted HeLa cells (lanes 4-6) demonstrating reduced EGF-induced Src activation as measured by Src autophosphorylation (Src-pY419) upon MICAL-L1-depletion.  
(B,C) Control (B) and MICAL-L1 KD (C) cells were stimulated with EGF for 15 minutes, fixed and labeled with Src-pY419 (green) and paxillin (red).  
Profile analysis of two individual focal adhesions demonstrates recruitment of active Src to paxillin-containing focal adhesions in control (B) but not MICAL-L1 KD cells (C).  
(D-G) Under serum starved (SS) conditions, total-Src (green) localizes to the perinuclear region (arrows) in control (D) and MICAL-L1 siRNA-treated cells (F).  
In response to EGF, Src translocates from the ERC to the plasma membrane in control cells (E) but is largely retained in the ERC in MICAL-L1 KD cells (G; arrows).  
(H-J) Cells were stimulated with EGF and stained for Src (green) and transferrin receptor (TfR; red).  
Note the increased overlap of Src and TfR at the ERC in MICAL-L1 KD cells (I; yellow arrows) compared to control (H).  
(J) ImageJ was used to quantify Src fluorescence in the TfR-positive ERC after EGF stimulation and one-way ANOVA showed that there was significantly more Src in MICAL-L1 KD compared to control (p<0.01)  
Error bar=S.E.M.  
Blue=DAPI, scale bar=10 μm.

**Figure 3** MICAL-L1 co-localizes with Src and focal adhesion proteins along circular dorsal ruffles (CDR) in human foreskin fibroblasts (BJ).  
(A-F) BJ cells were serum-starved (A-C) or stimulated with 15 ng/ml PDGF (D-F) for 10 min.
Immunofluorescence of Src (green) and MICAL-L1 (red) demonstrates their co-localization along CDR (arrows; see inset). (G-L) BJ cells were stimulated with PDGF and stained for MICAL-L1 (green) and pY397FAK (red; G) or pY118paxillin (red; J). Arrows depict co-localization along CDR. Arrowheads mark co-localization of MICAL-L1 and pY397FAK on macropinocytic-like vesicles. Blue=DAPI, scale bar=10 μm.

**Figure 4** MICAL-L1 regulates Src recruitment to CDR. (A-C) Control (A) and MICAL-L1 KD cells (B) were stimulated with PDGF for 10 min., fixed and stained with Src (green) and phalloidin to show F-actin (red). Src recruitment to CDR was then quantified using profile analysis (C). (D-J) Control (D-F) and MICAL-L1 KD cells (G-I) were stimulated with PDGF for 20 min. and stained with Rabankyrin-5 (green) and Rab5 (red) to mark macropinosomes (yellow arrows). Cells containing macropinosomes were manually counted in 100 cells/experiment (I). Error bar=S.E.M. Blue=DAPI, scale bar=10 μm.

**Figure 5** MICAL-L1 regulates PDGF-induced focal adhesion turnover (A-F) Representative images of control and MICAL-L1-depleted fibroblasts stimulated with PDGF for 10 min. and stained for vinculin (green) and Src (red). Arrows denote co-localization of Src and vinculin at CDR in control cells while arrowheads mark the ERC-localized Src in MICAL-L1-depleted cells. (G-L) Control and MICAL-L1-depleted fibroblasts were serum-starved (SS) (G and J) or stimulated with PDGF for 10 min. (H and K) or 15 min. (I and L). Focal adhesions are labeled with vinculin. (M) The number of focal adhesions in SS or PDGF-stimulated cells was quantified using ImageJ. Error bars=S.E.M from three independent experiments. Total number of cells and focal adhesions are shown in Figure 6E. For rescue, focal adhesions were quantified from 45 cells from three independent experiments (15/experiment). Tukey Test was used to calculate statistical significance between treatments. Number of focal adhesions were significantly (p<0.01) different between control ss and MICAL-L1 ss but not between control and MICAL-L1 rescue SS. There was also a significant
difference between control PDGF and MICAL-L1 PDGF. MICAL-L1 rescue was significantly less than MICAL-L1 KD but was also significantly different than control PDGF (p<0.05). Blue=DAPI, scale bar=10 μm.

**Figure 6** MICAL-L1-depletion impedes focal adhesion turnover and leads to increased focal adhesion size. (A-D) Images from Figure 5 were used to demonstrate size distribution quantification of focal adhesions in control and MICAL-L1-depleted BJ cells. Images were imported into ImageJ and fluorescence levels were set to a threshold to optimally depict focal adhesions. Focal adhesion area was calculated using the ‘measure particles function’ and size distributions were set to group focal adhesions into three size categories: small (1-5 μm²), medium (6-10 μm²) and large (11-30 μm²). (E) Breakdown of focal adhesion number and size distribution in serum starved (SS) and PDGF-stimulated cells. Data is presented as percent of focal adhesions within each area category for three independent experiments. Tukey Test was used to calculate statistical significance. For small focal adhesions, there were significantly less focal adhesions in PDGF treated MICAL-L1 KD cells compared to control. In contrast, MICAL-L1 KD cells had significantly more medium and large focal adhesions compared to control cells (p<0.01). Scale bar=10 μm. (F) Representative immunoblot of BJ cells stimulated with PDGF in the presence (lanes 1-4) or absence of MICAL-L1 (lanes 5-8). T-FAK, t-Src and actin were used as loading controls.

**Figure 7** MICAL-L1 is required for proper cell spreading on fibronectin and optimal integrin-induced Src activation. (A-D) Control (A) and MICAL-L1-depleted cells (B-D) were serum starved (SS) in suspension for 1 h and then plated onto 10 μg/ml fibronectin-coated coverslips for 90 min. and stained with phalloidin-488 (green) and vinculin (red). In spreading MICAL-L1-depleted cells, prominent short actin cables (B; arrows), polygonal cells with disorganized actin stress fibers (C) and dorsal actin arcs (D; arrowhead) were observed. Scale bar=10 μm. (E) Average cell area was quantified from three independent
experiments (control n=65, MICAL-L1 siRNA n=66) using Pascal LSM image examiner. Error bars= S.E.M. One-way ANOVA demonstrated a significant increase in MICAL-L1 KD cell area compared to control cells (p<0.01). (F) Average number of focal adhesions/cell were quantified from three independent experiments (control n=40, MICAL-L1 siRNA n=53). There were significantly more focal adhesions/cell in MICAL-L1 KD cells (p<0.01) (G) Representative immunoblot of BJ cells held in suspension or plated onto fibronectin-coated plates in the presence (lanes 1-4) or absence of MICAL-L1 (lanes 5-8). Arrow denotes Src band. T-FAK, t-Src and actin were used as loading controls.

**Figure 8** MICAL-L1 is required for cell migration. (A-F) BJ cells were grown to confluence on fibronectin-coated coverslips and a scratch wound was applied. Monolayers were washed and incubated in low-serum medium for the times indicated, fixed, and stained with phalloidin. Images were taken with a 10X objective and are representative of three independent experiments. Yellow line denotes final degree of wound closure. (G-I) Cells were wounded, allowed to migrate for 6 h and then stained for Src (G) and MICAL-L1 (H). Arrowhead marks co-localization along the leading edge. Arrows in the inset show co-localization along tubulo-vesicular structures. (J-N) Loss of MICAL-L1 impairs wound polarization. Cell monolayers were wounded and allowed to migrate for 6 h prior to fixation and then stained for phalloidin and GM130 (J,K) or vinculin (L,M). + and – denote proper polarization of the Golgi apparatus towards the wound, or lack of it, respectively. Representative images of three independent experiments are shown (J-M) and quantified (N, statistically significant p<0.05). Asterisk= direction of wound. Error bars=S.E.M. Blue=DAPI, scale bar=10 μm.

**Figure 9** EHD1 is required for EGF-induced Src translocation and activation in HeLa cells. (A-G) Control cells (A-C) and EHD1 siRNA cells (D-F) were stimulated with EGF and stained with Src (green) and TfR (red). EHD1-siRNA results in retention of Src in the ERC (overlap between Src and TfR; compare C to F). (G) The level of Src fluorescence in the ERC was quantified from three
independent experiments and found to be significantly higher in EHD1 KD cells (One-way ANOVA, p<0.01). (H) Immunoblot demonstrating impaired Src activation in EHD1-depleted cells (lanes 4-6) compared to control cells (lane 1-3). Pan-actin and t-Src were used as loading controls. Error bars=S.E.M. Blue=DAPI, scale bar=10 μm.

Figure 10 EHD1 is required for EGF-induced MICAL-L1 tubule vesiculation. (A-I) Compared to steady state (A-C) and serum starved cells (SS) (D-F), EGF treatment (G-I) induces increased recruitment of EHD1 to MICAL-L1-positive tubules (arrows) resulting in the cleavage and vesiculation of MICAL-L1-decorated recycling tubules. (J-N) EHD1-siRNA impairs vesiculation of MICAL-L1-decorated tubular endosomes in response to EGF. In control cells, EGF stimulation leads to a reduction in the total area of MICAL-L1-containing tubular endosomes (compare K to J; quantified in N). EHD1-siRNA results in a modest increase in the area of MICAL-L1-containing tubular endosomes in SS cells (L). In the absence of EHD1, EGF stimulation does not induce vesiculation of MICAL-L1-decorated tubular endosomes (M; quantified in N). Tubule area was quantified from three independent experiments (at least 30 cells/experiment). Tukey Test demonstrated that there were significant increases in MICAL-L1 tubular area in SS and EGF treated EHD1KD cells compared to control (p<0.01). No significant difference was observed between SS EHD1 KD and EGF- treated EHD1 KD cells. Error bars=S.E.M. Blue=DAPI, scale bar=10 μm.

Figure 11 Schematic model depicting the proposed roles of MICAL-L1 and EHD1 in mediating Src translocation from the ERC to the plasma membrane in response to growth factor or integrin stimulation.

Supplementary Figure 1 MICAL-L1-depletion in HeLa cells does not effect EGF binding capacity, EGFR internalization or EGFR activation. (A,B) Control-(blue bar) and MICAL-L1-siRNA-treated (orange bar) HeLa cells were labeled with EGF-Rhodamine as described in the materials and methods and EGF surface binding was quantified using flow cytometry (red=unlabeled, background
control). The histogram and graph are representative ones from 3 experiments, each done with ~10,000 cells. (C-F) Control (C,D) and MICAL-L1 KD (E,F) cells were serum starved (SS) and then pulsed with unlabeled EGF to stimulate EGFR internalization. Immunofluorescence demonstrates that EGFR internalization is similar in control (D) and MICAL-L1-siRNA cells (F). (G) Immunoblot analysis of EGFR autophosphorylation in control and MICAL-L1-siRNA cells (additional siRNA oligonucleotides are included to demonstrate specificity). MICAL-L1 KD has no effect on EGF-induced EGFR phosphorylation (compare lane 1-4 to 5-8).

**Supplementary Figure 2** MICAL-L1-depleted BJ cells show altered distribution but not expression of focal adhesion proteins at steady-state. (A) Immunoblot analysis demonstrating efficiency of MICAL-L1-depletion and expression of several focal adhesion proteins. (B-G) Distribution of active Src (B and C) vinculin (D and E) and paxillin (F and G) in MICAL-L1-depleted fibroblasts compared to control cells. Arrows mark the accumulation of Src in intracellular vesicles in MICAL-L1-depleted cells. Blue=DAPI Scale bar=10 μm.

**Supplementary Figure 3** Vinculin and phalloidin double-staining of CDR in MICAL-L1 KD cells and introduction of siRNA-resistant GFP-MICAL-L1 into MICAL-L1-depleted fibroblasts partially rescues focal adhesion number and turnover. (A-F) Control (A-C) and MICAL-L1 KD cells (D-F) were stimulated with PDGF for 10 minutes, fixed and stained with vinculin (green) and phalloidin (red) to mark CDR (yellow arrows). (G-J) MICAL-L1-depleted cells were electroporated with an siRNA-resistant construct, plated onto fibronectin, serum starved (G,H) and fixed or stimulated with PDGF for 15 min. (I,J) prior to fixation. GFP fluorescence denotes transfected serum-starved (G) and PDGF-stimulated (I) cells. Focal adhesions were identified by vinculin (H,J) staining and GFP-MICAL-L1 transfected cells are marked by an asterisk. Scale bar=10 μm.

**Supplementary Figure 4** MICAL-L1-depletion impairs PDGF-induced cell elongation that is partially rescued by siRNA-resistant GFP-MICAL-L1. (A,B)
Control and MICAL-L1-depleted cells were stimulated with PDGF for 30 min., fixed and stained with phalloidin-488 to mark the actin cytoskeleton (green). (C-E) MICAL-L1-depleted cells were electroporated with siRNA-resistant GFP-MICAL-L1, stimulated with PDGF, fixed and stained with phalloidin-Rhodamine (red). Arrows denote formation of single lamellipodia in migrating cells and transfected cell in E. Scale bar=10 μm. (F) Cell elongation was quantified from three independent experiments as described in materials and methods. N=45 cells/experiment/treatment. Error bar=S.E.M. Tukey Test demonstrated cell elongation was significantly impaired in MICAL-L1 KD cells (p<0.01). GFP-MICAL-L1 partially rescued cell elongation phenotype (significantly more elongated than MICAL-L1 KD cells but significantly different than control cells, p<0.01).
Figure 1

HeLa-Steady State

A. Src

B. MICAL-L1

C. Merge

Inset

D. Src-GFP

E. HA-MICAL-L1

F. Merge

SYF MEFs-Steady State
Figure 6

Threshold

A. Control

B. MICAL-L1siRNA

C. 15 min. PDGF

D. MICAL-L1siRNA

E. Focal Adhesion Area

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>MICAL-L1</th>
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<tr>
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Total focal adhesions: 4540.0, 8853.0, 1176.0, 5356.0

Total number of cells: 184.0, 161.0, 122.0, 100.0

F. Western Blot

Control siRNA

MICAL-L1 siRNA
Figure 7

A. Control vs. siRNA

B, C, D. MICAL-L1 siRNA

E. Cell area (μm²)

F. Focal Adhesions/Cell

G. Western Blot Analysis

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