Deletion of integrin linked kinase in endothelial cells results in defective RTK signaling caused by caveolin 1 mislocalization

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SUMMARY
Integrin linked kinase (ILK) connects the ILK-Pinch-Parvin complex with integrin adhesion sites. Because of the functional relevance of integrin-linked signaling for endothelial cell (EC) biology, we have explored this pathway in Ilk−/− embryonic stem (ES) cells differentiated into ECs and vessel-like structures. We have focused in particular on the mechanistic relevance of ILK-Pinch-Parvin complex-related signaling for EC development and tube formation. Our analysis revealed that the formation of vessel-like structures was strongly reduced in Ilk−/− ES cells and that this phenotype could be rescued by re-expression of ILK in ES cells. ECs were MACS sorted from wild-type (WT) and Ilk−/− ES cells and functional analysis using intracellular calcium imaging as the read-out yielded a complete lack of vascular endothelial growth factor- and epidermal growth factor-dependent responses. The possibility of a caveolin 1-related defect was investigated by transfecting WT and Ilk−/− ECs with a caveolin 1-EGFP fusion protein. Time-lapse microscopy showed that the prominent phenotype is due to altered dynamics of caveolin 1 and to a lack of positioning of caveolin 1 in the vicinity of the plasma membrane and that it is rescued by re-expressing ILK in the Ilk−/− ES cells. We also found that the defect is caused by the perturbed organization of microtubules and cortical actin filaments. Thus, ILK is required as a scaffold to allow actin-microtubule interactions and correct positioning of caveolin 1 close to the plasma membrane. This is crucial for signaling compartmentalization in ECs and explains the key role of ILK for EC development and function.

KEY WORDS: Integrin linked kinase (ILK), Endothelial cells, Tyrosine kinase signaling, Mouse

INTRODUCTION
Integrin linked kinase (ILK) is a ubiquitously expressed scaffold protein that functions as a central component and mediator of cell-extracellular matrix (ECM) interactions (Novak et al., 1998). ILK binds the cytoplasmic tail of β1 integrin and consists of three structural and functional domains (Dedhar and Hannigan, 1996): five ankyrin repeats at the N terminus, which allow interaction with PINCH; a Pleckstrin-homology (PH) domain; and a C-terminal kinase-like domain (Wickström et al., 2010b). By targeting of the PINCH-Parvin complex to integrin adhesion sites, ILK regulates the engagement and remodeling of the actin cytoskeleton downstream of integrin adhesion (Wickström et al., 2010a; Wickström et al., 2010b). Moreover, there is evidence for direct interactions of ILK with tyrosine kinase receptors (RTKs) via PINCH and the NCK2 adaptor protein (Tu et al., 1998). Thus, ILK links cell-matrix interactions with signals modulating remodeling of the cytoskeleton and is therefore involved in central cell biological processes such as cell adhesion, migration, proliferation, survival and differentiation (Hannigan et al., 2007).

ILK has been also reported to affect endothelial cell (EC) apoptosis, proliferation and migration as well as vascular development in vitro (Friedrich et al., 2004; Vouret-Craviari et al., 2004). In addition, defects in vascular development were observed in vivo in ILK-deficient mice (Friedrich et al., 2004). However, despite the EC-specific deletion of ILK, the mice died at an early embryonic stage and, therefore, the molecular defects underlying the observed delay of vascular development could not be determined (Friedrich et al., 2004). A recent publication in keratinocytes has provided new mechanistic insight into the cell biological role of ILK by illustrating its direct involvement in trafficking and its integration into the plasma membrane (Wickström et al., 2010a; Wickström et al., 2010b). Caveolae are present in most cell types, but are particularly abundant in ECs and are known to play a crucial role in EC biology (Cho et al., 2004). The principal component of caveolae in ECs is caveolin 1 and this molecule is known to cluster a great variety of different signaling molecules, e.g. RAS, nitric oxide, G-proteins and growth factors such as vascular endothelial growth factor (VEGF) (Krajewska and Maslowska, 2004). Caveolin 1 assists compartmentalization of signaling pathways by establishing specific lipid microdomains that act as specialized signaling hubs (Balijepalli et al., 2006; Saliez et al., 2008). Caveolin 1 also regulates multiple signaling cascades and to provide crosstalk with growth factors. Therefore, we hypothesized that the involvement of ILK in the formation of lipid rafts and/or caveolin 1 microdomains could be important for this crosstalk (Head et al., 2006; Saliez et al., 2008). Integrin-based adhesion has been shown to regulate multiple signaling cascades and to provide crosstalk with growth factors. Therefore, we hypothesized that the involvement of ILK in the formation of lipid rafts and/or caveolin 1 microdomains could be important for this crosstalk (Head et al., 2005; Head et al., 2006). In order to understand the potential relevance of ILK for EC biology, we have investigated EC development and function in Ilk−/− embryonic stem (ES) cells. Our study revealed strongly reduced formation of vessel-like structures in Ilk−/− embryoid bodies (EBs) because of defective EC signaling. This striking alteration is due to perturbed caveolin 1 positioning in vicinity of the plasma membrane.
MATERIALS AND METHODS

Cell culture

The mouse ES cell lines D3 (wild type) and Ilk−/− were derived and maintained in culture in hanging drops (Malan et al., 2010). Time after plating was indicated as (5+7) and (5+14) days.

Immunohistochemistry and detection of endothelial cells and vessels

EBs were fixed with 4% paraformaldehyde (PFA) and MACS-sorted ECs (M-ECs) were stained with the antibody rat anti-mousePECAM-1 (CD31) (1:800; Pharmingen, San Diego, CA, USA). Other antibodies/markers used were: rabbit polyclonal anti-collagen IV (1:500; Acris Antibodies), mouse monoclonal anti-perlecan (1:500; Biotrend, Köln, Germany), mouse monoclonal anti-fibronectin (1:500; Sigma-Aldrich, Munich, Germany), polyclonal rabbit anti-laminin (1:500; Sigma-Aldrich), rabbit polyclonal PLCy1 (1:50; abcam, Cambridge, UK), rat anti-mouse flk-1 (1:100, BD Pharmingen, Erembodegem Belgium), Alexa Fluor 488 Phalloidin (1:40; Molecular Probes Invitrogen, Karlsruhe, Germany), mouse monoclonal β-tubulin (1:500; Sigma-Aldrich), rabbit polyclonal anti-caveolin 1 (1:500; BD Pharmingen), wheat germ agglutinin conjugate Alexa Fluor 488 (1:500; Molecular Probes Invitrogen). Secondary antibodies were: Cy3- (or Cy2-) conjugated goat anti-rabbit (or goat anti-mouse) Ig (1:1500 and 1:500; Dianova, Hamburg, Germany).

Magnet-associated cell sorting (MACS)

Differentiated ES cells (5–7 days) were dissociated with Accutase (PAA Laboratories, Linz, Austria). The single cells were stained with an endothelial-specific marker, rat anti-mouse-PECAM-1 (also known as CD31), and MACS sorted as previously described (Schmidt et al., 2004). For analysis, the number of cells in 40 defined areas was calculated using a 40× objective on an Axiohot microscope (Zeiss Microimaging, Gottingen, Germany).

Proliferation assay and apoptosis assay

Wild-type and Ilk−/−EBs were fixed and stained with rat anti-PECAM-1 as described above. Proliferating cells were detected with the primary antibody rabbit anti-mouse Ki67 (1:150, pAb; Dianova) and apoptotic cells with a rabbit anti-active caspase 3 (1:500, BD Pharmingen). The number of Ki67- or caspase 3-positive cells within 50 randomly chosen vessel-like tubes was counted as proliferating or apoptotic M-ECs (Müller-Ehmsen et al., 2006).

Morphological analysis, apotome and confocal microscopy

The distribution pattern of extracellular matrix proteins, caveolin 1 and cytoskeletal components, as well as live images, were analyzed by confocal microscopy using the LSM 510 META Zeiss microscope (Zeiss Microimaging) or by an inverted confocal laser scanning microscope (Nikon Eclipse Ti). Alternatively, image stacks were acquired with the fluorescence microscope equipped with the ApoTome (Axiovert 200A, Zeiss Microimaging). Live images were acquired with the confocal laser scanning microscope with one image every second for 50 seconds in total. The analysis of velocity was carried out using a video image J 1.37v (Plug-In: Particle Analysis/Manual Tracking). For every picture, the same external calcium ([Ca2+]i) indicator Fura 2 am (5 µM; Molecular Probes Invitrogen) for 10 minutes at room temperature. The bath solution contained: 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 10 mM HEPES and 10 mM glucose. The emitted fluorescence was monitored using a charge-coupled device cooled camera (TILL Photonics, Planegg, Germany) coupled with an inverted microscope (Axiovert 200M, Zeiss Microimaging). The emission data were analyzed using the Vision software package (TILL Vision 4.0, TILL Photonics). Results are displayed as 340 nm/380 nm ratios after background subtraction. n is the number of cells tested. A [Ca2+]i increase of <10% was considered to be ‘no response’. M-ECs were in some experiments treated with 2% methyl-beta-cyclodextrin (MbetaCD) for 2 hours at 37°C. All drugs used were from Sigma-Aldrich except for epidermal growth factor (EGF) and VEGF (both from PAN-Biotech) and the PLC activator m3M3FBS (Calbiochem, Merck Millipore, Darmstadt, Germany).

Protein detection and western blot analysis

For western blotting, samples were submitted to SDS-PAGE and proteins were transferred to PVDF membrane and incubated with specific antibodies. Immunoreactive proteins were detected by the enhanced chemiluminescence detection system (Amersham Biosciences Europe, Freiburg, Germany) and normalized to the actin content. VEGF (PAN Biotech, Aidenbach, Germany) stimulation was achieved by treating the cells for 6 hours with low serum medium (1%), then 20 ng/ml VEGF (PAN Biotech) was applied for 4 minutes. We used a concentration that has been reported to be physiologically relevant in inducing vascular-angiogenesis (Schmidt et al., 2005; Hagedorn et al., 2004); this concentration also induced a clear Ca2+ release from the sarcoplasmic reticulum. Densitometry analysis was carried out using Image software (NIH) and normalized to the actin content. Antibodies used were: rabbit polyclonal anti-caveolin-1 (1:500; Acris Antibodies), rabbit polyclonal anti-GFP (1:500; Santa Cruz Biotechnology, Heidelberg, Germany), rabbit polyclonal anti-VEGFR2 and anti-PhosphoY1054-1059VEGFR2 (1:2000; Abcam), mouse monoclonal anti-MAP kinase activated (1:1000; Sigma-Aldrich), rabbit polyclonal anti-MAP kinase (1:1000; Upstate, Merk Millipore, Billerica, MA, USA), rabbit polyclonal anti-phosphoPLC-γ1 (Try783) antibody (1:500; Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal anti-PLCγ1 (1:1000; Cell Signaling Technology), mouse monoclonal anti-actin (1:4000; Chemicon Millipore, Billerica, MA, USA).

Migration assay

Migration assay was performed in a modified Boyden chamber. The total number of PECAM-positive migrated cells was counted as well as the number of migrated M-ECs (n=6) as described previously (Schmidt et al., 2004).

Generation of transgenic ES cell clones

A pCL-MFG fusion plasmid containing the ILK-EGFP cDNA (provided by R. Fässler, Max Planck Institute of Biochemistry, Department of Molecular Medicine, Martinsried, Germany). By cutting the construct with EcoRI/NotI, the EGFP cDNA was excised and subsequently cloned into ABD 15-24 pEGFP-N3/β-actin (BD Biosciences Clontech, Heidelberg, Germany) with ABD 15-24-EGFP excised. The resulting β-actin-ILK-EGFP fusion construct was used for electroporation of ILK-ES cells.

Generation of the caveolin 1-EGFP fusion protein

The caveolin 1 cDNA (Homo sapiens, PubMed BC082246) was cloned in-frame upstream of the EGFP cDNA of the pEGFP-1 plasmid (BD Biosciences Clontech), in the BamHI restriction site. The CAG promoter from pDRIVE-CAG (InvivoGen, San Diego, CA, USA) was cloned into the SalI/Sall restriction site of the caveolin 1-EGFP fusion plasmid.

Ca2+ imaging

M-ECs were loaded with the intracellular calcium ([Ca2+]) indicator Fura 2 am (5 µM; Molecular Probes Invitrogen) for 10 minutes at room temperature. The bath solution contained: 140 mM NaCl, 5.4 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2, 10 mM HEPES and 10 mM glucose. The emitted fluorescence was monitored using a charge-coupled device cooled camera (TILL Photonics, Planegg, Germany) coupled with an inverted microscope (Axiovert 200M, Zeiss Microimaging). The emission data were analyzed using the Vision software package (TILL Vision 4.0, TILL Photonics). Results are displayed as 340 nm/380 nm ratios after background subtraction. n is the number of cells tested. A [Ca2+]i increase of <10% was considered to be ‘no response’. M-ECs were in some experiments treated with 2% methyl-beta-cyclodextrin (MbetaCD) for 2 hours at 37°C. All drugs used were from Sigma-Aldrich except for epidermal growth factor (EGF) and VEGF (both from PAN-Biotech) and the PLC activator m3M3FBS (Calbiochem, Merck Millipore, Darmstadt, Germany).
RESULTS
ILK deficiency results in strongly reduced differentiation into endothelial tube-like structures

First, we investigated vascular development in wild-type (WT) and Ilk–/– EBs based on the morphological characteristics of endothelial structures at different time points of development. We found a strongly reduced number of PECAM-positive endothelial tubes in the Ilk–/– EBs (Fig. 1A) compared with WT controls; Ilk–/– ECs were preferentially assembled in clusters (Fig. 1A, middle). Quantitative analysis of the main types of vessel structures (Fig. 1B; see Materials and methods) revealed that WT EBs possessed similar percentages of cells, clusters and vessel-like structures (25%, 33% and 41%, respectively) at 5+7 days (5+7d), whereas in Ilk–/– EBs a higher proportion of single cells (75.3%, 22.2% and 2.5% for cells, clusters and vessels, respectively) was detected (Fig. 1C). Similarly, at the late differentiation stage (5+14d) lower numbers of vessel-like structures (10%, 37.5% and 52.5% for cells, clusters and vessels, respectively, at 5+14d), suggesting a partial defect of vascular development in Ilk–/– EBs, we generated stable rescue ES cell lines, in which the CAG promoter drives expression of ILK. In these EBs, the percentage of vessel-like structures increased (30%, 46.9% and 19.3% for cells, clusters and vessels, respectively, at 5+7d; and 10%, 37.5% and 52.5% for cells, clusters and vessels, respectively, at 5+14d), suggesting a partial rescue (see also Fig. 1A, right-hand panel; Fig. 1C).

In order to understand better the observed differences between WT and Ilk–/– EBs, we assessed the cell biological properties of the ECs in more detail. We found a significant increase in the rate of apoptosis in Ilk–/– ECs at 5+7d (WT: 32.2±12.9, n=8 differentiated EBs; Ilk–/–: 191.0±38.2, n=8), as well as at 5+14d of development (WT: 17.2±3.6, n=8; Ilk–/–: 124.2±33.6, n=8). The proliferation rate was very similar in WT and Ilk–/– ECs at the early stage of development (WT: 43.8±6.4, n=8; Ilk–/–: 38.8±4.2, n=8), whereas it was increased in the Ilk–/– cells at the late stage (WT: 16.9±4.5, n=8; Ilk–/–: 59.8±9.5, n=8). The migration rate did not differ at 5+7d (WT: 59.2±6.6, n=4; Ilk–/–: 65.0±13.1, n=4), whereas in 5+14d EBs a higher migration of Ilk–/– PECAM-positive ECs was seen (WT: 55.0±4.7, n=4; Ilk–/–: 96.0±16.8, n=4). Thus, our data show that ILK deficiency results in a striking impairment of vessel formation. The observed discrepancy between proliferation and apoptosis rates at the late differentiation stage could explain the decreased vessel formation in the Ilk–/– EBs.

VEGF signaling is defective in Ilk–/– ECs

It is well known that VEGF signaling plays a key role in EC biology and vascular development. The observed phenotype in the Ilk–/– EBs prompted us to investigate the expression and distribution pattern of VEGFR2 (KDR – Mouse Genome Informatics) using immunohistochemistry. This analysis did not reveal obvious differences between WT and Ilk–/– MACS-sorted endothelial cells (M-ECs) (Fig. 2A). We also quantified VEGF2 expression by western blotting and found comparable levels in WT and Ilk–/– M-ECs (Fig. 2B) [arbitrary optical density (OD)=0.99±0.002 in WT and 0.99±0.01 in Ilk, n=3]. As normal expression levels of the receptor cannot exclude functional defects, we next assessed the phosphorylation of VEGFR2 in response to VEGF (20 ng/ml). We found that phosphorylation of the receptor occurred in both WT and Ilk–/– M-ECs; however, phosphorylation levels before and after stimulation were higher in the mutant cells compared with WT cells (relative expression, arbitrary OD=0.7±0.08 in WT and 0.94±0.08 in Ilk–/–; 1.01±0.07 in Ilk–/– with VEGF; 1.2±0.04 in Ilk–/– with VEGF, n=3; *P<0.05), suggesting alterations in the basal regulation of VEGFR2 activity (Fig. 2C). Because of the preserved VEGF-mediated phosphorylation of the VEGFR2 in Ilk–/– M-ECs, we suspected downstream signaling defect(s) and explored the key pathways, namely ERK1/2 (MAPK3/1) and PLC-γ (PLCG1) activation. Similar to VEGFR2, ERK1/2 was phosphorylated in WT and Ilk–/– M-ECs (Fig. 2D), but, again, the latter displayed higher phosphorylation levels prior to and post VEGF stimulation (arbitrary OD=0.61±0.03 in WT, 0.81±0.04 in WT with VEGF, 0.78±0.03 in Ilk–/– and 0.98±0.07 in Ilk–/– with VEGF, n=3; *P<0.05). The overall levels of ERK1/2 remained stable in all conditions (arbitrary OD=1.02±0.01 in WT, 1.01±0.05 in WT with VEGF, 1.03±0.05 in Ilk–/– and 0.99±0.04 in Ilk–/– with VEGF, n=3; supplementary material Fig. S3H).

We determined next the downstream signaling pathway in WT and Ilk–/– M-ECs using single-cell [Ca2+]i imaging. The majority (67.1%) of WT M-ECs responded to VEGF (20 ng/ml, applied via a micropipette) with an increase of [Ca2+]i (Fig. 2E, left-hand panel); resting [Ca2+]i was augmented by 57.3±5.8% (n=49, Fig. 2E, right-hand panel). By contrast, almost all (99.1%, n=90) Ilk–/– M-ECs
lacked a clear \([\text{Ca}^2+]_i\) response upon application of VEGF (Fig. 2E, middle; the average change in \([\text{Ca}^2+]_i\) was \(-4.8\pm1.1\% (n=90, \text{Fig. 2E, right-hand panel}). These data suggest that VEGFR2-induced signaling is severely impaired in \(\text{Ilk}^{-/-}\) M-ECs and we therefore explored in more detail the underlying defect.

**ILK deficiency causes defective tyrosine kinase signaling**

Because of the lack of an increase in \([\text{Ca}^2+]_i\) upon VEGF application in \(\text{Ilk}^{-/-}\) M-ECs, we wondered whether similar signaling defects also occurred with other RTKs in these cells. Indeed, EGF (20 ng) also failed to augment \([\text{Ca}^2+]_i\) in \(\text{Ilk}^{-/-}\) M-ECs in contrast to WT M-ECs; 72.4\% of WT cells (\(n=21\)) showed an EGF-induced increase of \([\text{Ca}^2+]_i\) by \(52.2\pm10.8\%\), whereas 58 out of 59 \(\text{Ilk}^{-/-}\) cells did not respond to EGF and this is also reflected by the marginal average increase of \([\text{Ca}^2+]_i\) by \(2.4\pm0.6\%\) (Fig. 3A). Importantly, we could also demonstrate that the defective response to VEGF and EGF was at least partially restored in rescued \(\text{Ilk}^{-/-}\) M-ECs (supplementary material Fig. S1A,B). In fact, the majority of rescued cells (73.3\%, \(n=33\)) responded to EGF and a lower percentage (30.7\%, \(n=11\)) to VEGF; the percentage of \([\text{Ca}^2+]_i\) increase amounted to \(60.1\pm3.1\% (n=33)\) and \(34.1\pm5.4\% (n=11)\), respectively (supplementary material Fig. S1C).

Next, we investigated whether other PLC-\(\gamma\)-dependent signaling pathways were also affected by analyzing \(G_\alpha\)-coupled agonists. When stimulating M-ECs with bradykinin (100 nM), most of the WT (91.4\%, \(n=64\)) and \(\text{Ilk}^{-/-}\) (67.4\%, \(n=58\)) cells responded with a transient \([\text{Ca}^2+]_i\) response (Fig. 3B) of comparable magnitude. Similar results were also obtained with acetylcholine (ACh; 10 \(\mu\)M), which showed preserved activation of the \([\text{Ca}^2+]_i\) response in \(\text{Ilk}^{-/-}\) M-ECs (WT: 73.5\% of responders, \(n=36\); \(\text{Ilk}^{-/-}\): 69.5\% of responders, \(n=16\)). To pinpoint more precisely the signaling defect, in particular whether it occurred at the receptor level or downstream of the receptor, we used first the direct PLC activator m-3M3FBS. Application of this compound led to an increase of \([\text{Ca}^2+]_i\) in both WT and \(\text{Ilk}^{-/-}\) M-ECs (Fig. 3C), suggesting that PLC-\(\gamma\) and its related downstream signaling components were functional upon direct activation; the percentage of \([\text{Ca}^2+]_i\) increase was \(24.7\pm1.6\%\) in WT cells (\(n=29\)) and \(29.3\pm4.8\%\) in \(\text{Ilk}^{-/-}\) cells (\(n=19\)) (Fig. 3C, right-hand panel) (WT: 74.4\% of responders; \(\text{Ilk}^{-/-}\): 76\% of

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**Fig. 2. VEGF signaling is defective in \(\text{Ilk}^{-/-}\) M-ECs.** (A) VEGFR2 distribution in WT and \(\text{Ilk}^{-/-}\) M-ECs (anti-VEGFR2 staining, red). (B-D) Western blots of VEGFR2 (B), its phosphorylated form (pVEGFR2) (C) and ERK1/2 as well as its phosphorylated form (pERK) (D) in WT and \(\text{Ilk}^{-/-}\) M-ECs. The densitometric analysis of these western blots is shown below (\(n=3, *P<0.05\); see also supplementary material Fig. S3H). (E) VEGF (20 nM) evokes an increase of \([\text{Ca}^2+]_i\), in WT (left) but not \(\text{Ilk}^{-/-}\) (middle) M-ECs, whereas thapsigargin (TH; 1 \(\mu\)M) elevates \([\text{Ca}^2+]_i\), also in M-ECs (middle); representative \([\text{Ca}^2+]_i\) traces are shown, each color labels the 340/380 nm ratio in an individual cell over time. Right: statistical analysis of the percentage increase of \([\text{Ca}^2+]_i\), upon drug application. Error bars represent s.e.m. Scale bar: 5 \(\mu\)m.

**Fig. 3. Tyrosine kinase signaling is defective in \(\text{Ilk}^{-/-}\) M-ECs.** (A) EGF (20 nM) evokes an increase of \([\text{Ca}^2+]_i\), in WT but not \(\text{Ilk}^{-/-}\) M-ECs. (B) Bradykinin (Bk, 100 nM) elevates \([\text{Ca}^2+]_i\), in both WT and \(\text{Ilk}^{-/-}\) M-ECs. (C) Direct activation of PLC with m-3M3FBS (25 \(\mu\)M) also augments \([\text{Ca}^2+]_i\), in WT and \(\text{Ilk}^{-/-}\) M-ECs; each color labels the 340/380 nm ratio in an individual cell over time. For A-C, statistical analyses of the percentage \([\text{Ca}^2+]_i\), increase upon drug application in comparison to control conditions is shown on the right. (D) The cellular distribution of PLC-\(\gamma\) (green) is assessed by immunohistochemistry. (E) Expression analysis of PLC-\(\gamma\) and its phosphorylated isoform (Tyr783) is performed by western blotting (left) and quantified by densitometry (right). Error bars represent s.e.m. Scale bar: 40 \(\mu\)m. See also supplementary material Fig. S1 and Fig. S3G.
responders). We next assessed the PLC-γ distribution pattern in WT and Ilk<sup>−/−</sup> M-ECs by immunocytochemistry and found that it was unchanged (Fig. 3D). Moreover, protein expression analysis of PLC-γ did not reveal significant differences between WT and Ilk<sup>−/−</sup> (Fig. 3E; supplementary material Fig. S3G). In addition, our experiments revealed that VEGF application lead to the phosphorylation of PLC-γ at the Tyr 783 site in WT and Ilk<sup>−/−</sup> M-ECs, indicating intact activation (arbitrary OD pPLC=1.02±0.18 in WT and 1.11±0.24 in WT with VEGF; 1.12±0.18 in Ilk<sup>−/−</sup> M-ECs with VEGF; n=4; Fig. 3E); the respective numbers are also significantly different (paired t-test WT versus WT with VEGF: P=0.0117; Ilk<sup>−/−</sup> versus Ilk<sup>+</sup> with VEGF: P=0.0033). Thus, our experiments showed that ILK plays a crucial role for RTK-dependent signaling in M-ECs.

**Caveolin 1 distribution is altered in Ilk<sup>−/−</sup> M-ECs**

ILK has been recently reported to play a key role in caveolae formation. As the components of the VEGF/PLC-γ/PtdIns(4,5)P<sub>2</sub> (PIP2) signaling axis are clustered within caveolae in ECs and earlier experiments underscore the crucial role of the clustering of signaling components, we explored the subcellular distribution of components within caveolae. For this purpose, we used double immunohistochemical analysis with the plasma membrane marker wheat germ agglutinin and an antibody against caveolin 1. Caveolin 1 is the main protein component of caveolae in ECs and is essential for caveolae formation (Drab et al., 2001). The stainings illustrated that caveolin 1 is associated with the plasma membrane in WT (Fig. 4A, left-hand panel, see arrow), but not in Ilk<sup>−/−</sup> M-ECs (Fig. 4A, right-hand panel). This important finding was corroborated by electron microscopy. Even though the absolute number of typical caveolar structures is relatively low in cultured M-ECs, we could clearly identify these in close vicinity to the plasma membrane in WT cells, whereas this was not observed at all in any of the Ilk<sup>−/−</sup> M-ECs (60 cells in each preparation were analyzed; Ilk<sup>−/−</sup>: n=3 preparations; rescued Ilk<sup>−/−</sup>: n=2 preparations; WT: n=3 preparations) (Fig. 4B). Interestingly, western blotting experiments showed that the overall expression level of caveolin 1 did not significantly differ between WT and Ilk<sup>−/−</sup> M-ECs (WT: 1.0±0.18 arbitrary OD, n=5; Ilk<sup>−/−</sup>: 0.95±0.16, n=4; P=0.7; Fig. 4D). In addition, even transfection with the caveolin 1-EGFP fusion protein (see also below) did not change the overall expression of caveolin 1 in the cells (WT-transfected: 1.01±0.06 arbitrary OD, n=7; ILK-transfected: 1.02±0.08, n=5; P=0.7). Because of the lack of caveolin 1 association with the plasma membrane in Ilk<sup>−/−</sup> M-ECs, we investigated whether disruption of caveolin 1 assembly and caveolin 1 microdomain formation in WT M-ECs could reproduce the functional defects observed in the Ilk<sup>−/−</sup> M-ECs. For this purpose, we used beta cyclodextrin (MbetaCD); this compound is a known disruptor of lipid rafts and acts via depletion of cholesterol causing malpositioning of membrane protein complexes (Barbuti et al., 2004; Jang et al., 2001). In agreement with our hypothesis, MbetaCD (2%) prevented VEGF-induced increase of [Ca<sup>2+</sup>]i in WT M-ESCs (n=11) (Fig. 4C), whereas, as would be expected (see also Fig. 3B), the bradykinin response was preserved in the MbetaCD-treated Ilk<sup>−/−</sup> M-ESCs (Fig. 4C); the percentage of [Ca<sup>2+</sup>]i increase was 55.5±6.2% in WT cells treated with MbetaCD (n=11) with 42.4% of responders, whereas none of the cells tested responded to VEGF stimulation. These data suggest that intact caveolin 1 microdomains in the vicinity of the plasma membrane are required for functional RTK signaling and that deletion of ILK leads to defective caveolin 1 positioning and caveolae formation.

**Altered intracellular trafficking and subcellular distribution of caveolin 1 in Ilk<sup>−/−</sup> M-ECs**

Because of these findings, we investigated the formation and subcellular localization of caveolae in more detail. To this end, we transfected M-ECs with a caveolin 1-EGFP fusion protein (Fig. 4E). This colocalized with endogenous caveolin 1 in both WT and Ilk<sup>−/−</sup> M-ECs (Fig. 4F, red). We could identify a clear difference in the localization of the caveolin 1-EGFP fluorescence between WT and Ilk<sup>−/−</sup> cells; caveolin 1 had membrane localization in WT cells (Fig. 4H, upper panel, arrows), whereas this was lost in Ilk<sup>−/−</sup> cells (Fig. 4H, lower panel, arrows). This striking difference in the subcellular distribution of caveolin 1 was also confirmed when
acquiring with live imaging apotome sections of whole M-ECs. The caveolin 1-EGFP signal was preferentially close to the cell membrane in WT (Fig. 5A, arrows), but was in the center of cells in Ilk–/– M-ECs (Fig. 5B, arrows). The idea that ILK determines caveolin 1 formation and its subcellular positioning was further corroborated by analyzing the caveolin 1-EGFP-positive vesicles. In WT M-ECs (n=41 vesicles from four cells), two types of motility are seen: yellow and blue arrows indicate fast long-distance motility in the periphery between the plasma membrane and the cytosol (dark-blue arrow), whereas white arrows indicate slow movement at the center or close to the plasma membrane (asterisk). In Ilk–/– M-ECs (n=42 vesicles from four cells), the red arrow indicates the typical static movement pattern of fusion proteins in the cytosol and beneath the plasma membrane. (D) Left: average velocities of fusion proteins in WT, Ilk–/– and rescue M-ECs. Right: distribution of the different velocities (0 to 0.8 µm/second). Error bars represent s.e.m. The images shown are snapshots taken from supplementary material Movies 1 and 2. Scale bars: 20 µm. See also supplementary material Fig. S2 and Fig. S3E,F.

Deletion of ILK affects the organization of actin filaments and microtubules

Because of the well-known role of ILK for cytoskeletal integrity and its importance for the trafficking of caveolin 1 to the plasma membrane (Wickström et al., 2010a; Wickström et al., 2010b), we explored whether this was also in ECs mechanistically linked to an impaired interaction of microtubules (MTs) with the cortical actin network. We investigated the distribution of actin and MTs in Ilk–/– cells and determined whether alterations in the architecture of the cytoskeleton could be detected. In WT cells, the MTs exhibited a clear distribution pattern extending from the center of the cells to sub-plasma membrane regions (Fig. 6A, left-hand panel). By contrast, the MTs in Ilk–/– cells (Fig. 6A, right-hand panel) revealed an altered orientation with dense packs of MTs being positioned in the center of M-ECs; in addition, the peripheral density of MTs was decreased. Importantly, we also found that the MT tips in Ilk–/– cells were not able to contact and interact with the cortical actin network (Fig. 6B, right-hand panel), whereas the well-organized MTs of WT cells extensively aligned with the cortical actin at the cell periphery (Fig. 6B, left-hand panel, arrows). Together, these data indicate that the interaction of MTs...
Here, we demonstrate that deletion of ILK results in a strong reduction of endothelial tube formation. This prominent EC phenotype is caused by defective trafficking of caveolin 1 to the plasma membrane resulting in defective VEGF signaling. This disturbance of RTK signaling is due to changes in MTs and cortical actin organization giving rise to the destabilization of caveolin 1 microdomains.

Ilk–/– EBs display a striking reduction of endothelial tube formation with alterations of EC proliferation, apoptosis and migration. This phenotype was, as demonstrated by rescue experiments, clearly related to the ILK deficiency. Because key events of EC biology are regulated by VEGF signaling, we have investigated expression and function of VEGF-coupled receptor 2 (VEGFR2) and downstream signaling pathways (Bhattacharya et al., 2009). Ca2+ imaging was employed to explore VEGF signaling in single M-ECs and revealed that there was no VEGF-induced [Ca2+]i response in mutant cells. This proved to be a more general defect of RTK signaling as EGF was also unable to mobilize [Ca2+]i, in these cells. Further experiments revealed that VEGFR2 is expressed and functional and that the signaling defect resides downstream. Interestingly, VEGFR2 and ERK1/2 displayed increased basal phosphorylation levels and this is in full agreement with earlier reports on the effects of MbetaCD treatment (Barbuti et al., 2004) or inhibition of ILK activity (Ruiz-Torres et al., 2006). Because of the intact phosphorylation of VEGFR2 and of PLC-γ upon VEGF stimulation and the preserved intracellular Ca2+ handling in Ilk–/– cells, we suspect that defective crosstalk between signaling components, most likely between PLC-γ and PI2, underlies the observed phenotype. In fact, in ECs these signaling components are clustered within caveolin 1-enriched microdomains and their disruption inhibits PI2 turnover. (Jang et al., 2001; Pike and Miller, 1998). Recently, it has been also shown that changes in caveolae density and function, or disruption of the cytoskeletal integrity perturb the compartmentalization of PI2 signals (Cui et al., 2010). Future experiments are needed to explore the signaling defect in more detail; in particular, activation of PLC-γ and its crosstalk with PI2 should be assessed using biochemical in vitro activity assays. Similarly, the distribution and concentration of PI2 in the plasma membrane also needs to be explored with and without VEGF activation.

Recently, ILK has been reported to be involved in caveolae organization in keratinocytes (Wickström et al., 2010a). Our present findings reveal that ILK plays a crucial role for the correct subcellular positioning of caveolin 1 in close vicinity of the plasma membrane of M-ECs and illustrate the consequences of its deletion for cytoskeletal integrity, cellular signaling and EC biology. We also mimicked the defective VEGF signaling found in Ilk–/– M-ECs by the treatment of WT cells with MbetaCD and these experiments underscore the specific role of lipid rafts in the organization of this signaling pathway. These findings are in line with earlier reports, in which PLC-γ phosphorylation was found to be preserved despite the disruption of plasma membrane microdomains, whereas PI2 function was reported to be strongly dependent on their clustering and on the integrity of the cytoskeleton (Jang et al., 2001). Although G protein-coupled receptors are known to be coupled with caveolae (Razani et al., 2002), these receptors are affected in a different fashion by caveolar malfunction. In fact, disruption of caveolae by MbetaCD in adult cardiomyocytes affects the β2- but not the β1-adrenoceptor-mediated response (Calaghan et al., 2008), possibly explaining why neither acetylsalicylic nor bradykinin signaling was affected in Ilk–/– M-ECs.
As the signaling defects observed in Ilk−/− M-ECs were associated with caveolin 1 microdomains, we subsequently explored caveolin 1 positioning and caveolea formation using a variety of cell biological assays. Electron microscopy and high-resolution fluorescence microscopy revealed that Ilk−/− M-ECs lacked caveolin 1 at the plasma membrane and sites of caveolea formation, respectively. This was corroborated by time-lapse microscopy of fluorescence-labeled caveolin 1, the central protein component of caveolae (Drab et al., 2001). We observed a clear lack of caveolin 1 at the plasma membrane and sites of caveolea formation. Rapid trafficking of caveolin 1 between the cytosol and the plasma membrane was observed in WT M-ECs, which is in accordance with previous studies and is associated with the high turnover of this protein in the cytosol (Mundy et al., 2002). This was further corroborated in our rescue M-ECs, in which re-expression of ILK restored caveolin 1 particle movement to that observed in WT cells. By contrast, a strong reduction of caveolin 1 transport to the cell membrane could be observed in Ilk−/− M-ECs. Also, in keratinocytes caveolin 1 distribution was found to be dependent on ILK; in addition, its impact on MT stability via mDia1 DIAPI – Mouse Genome Informatics) and IQGAP1 has been shown (Wickström et al., 2010a). MTs, in concert with the cortical actin network, are assumed to act as tracks for caveolin 1-positive vesicles, and MT-cortical actin crosstalk is required to enable the transfer of caveolin 1 via cortical actin to the plasma membrane, resulting in the formation of caveolea (Mundy et al., 2002; Wickström et al., 2010a). In Ilk−/− M-ECs, we found abnormal organization of the actin cytoskeleton and MT network causing reduced MT-actin interactions. To confirm that the defects in the transport of caveolin 1 observed by time-lapse microscopy resulted from abnormal MT-actin crosstalk, we inhibited actin polymerization using latrunculin. Treatment of WT M-ECs with latrunculin led to disruption of the actin cytoskeleton as well as re-distribution of caveolin 1 and this was reminiscent of the findings in the Ilk−/− M-ECs. By contrast, depletion of intracellular Ca2+ stores with caffeine did not strongly alter subcellular caveolin 1 dynamics. These data support the view that ILK acts in ECs as a direct scaffolding protein for actin and MT organization, and that ILK-dependent maintenance of cytoskeletal integrity is crucial for the transport, positioning and turnover of caveolin 1 in close vicinity of the plasma membrane; these findings also support those recently reported in keratinocytes (Wickström et al., 2010a). Thus, our data demonstrate that ILK in ECs, through its regulation of caveolin 1 movement and positioning, directly interferes with specific cellular signaling pathways, in particular RTK-mediated signaling, and that intact RTK signaling requires the precise spatial positioning of downstream signaling components.

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Competing interests statement
The authors declare no competing financial interests.

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