

VE-cadherin interacts with cell polarity protein Pals1 to regulate vascular lumen formation

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Abstract

Blood vessel tubulogenesis requires the formation of stable cell-to-cell contacts and the establishment of apico-basal polarity of vascular endothelial cells. Cell polarity is regulated by highly conserved cell polarity protein complexes such as the Par3-aPKC-Par6 complex and the CRB3-Pals1-PATJ complex, which are expressed by many different cell types and which regulate various aspects of cell polarity. Here we describe a functional interaction of VE-cadherin with the cell polarity protein Pals1. Pals1 directly interacts with VE-cadherin through a membrane-proximal motif in the cytoplasmic domain of VE-cadherin. VE-cadherin clusters Pals1 at cell-cell junctions. Mutating the Pals1-binding motif in VE-cadherin abrogates the ability of VE-cadherin to regulate apico-basal polarity and vascular lumen formation. In a similar way, the deletion of the Par3 binding motif at the C-terminus of VE-cadherin impairs apico-basal polarity and vascular lumen formation. Our findings indicate that the biological activity of VE-cadherin in regulating endothelial polarity and vascular lumen formation is mediated through its interaction with the two cell polarity proteins Pals1 and Par3.

Introduction

Many organs are composed of sheets of cells which are wrapped into tubes that can form either simple pipes such as the intestine or the kidney, but that can also form an extensive network of tubes such as the tracheal system of invertebrates or the blood and lymphatic vasculature of vertebrates (Lubarsky and Krasnow, 2003). The inner surfaces of these tubes are lined with epithelial cells or endothelial cells. Both cell types are highly polarized with a well developed apico-basal membrane polarity. The apical domain faces the lumen of the tube, the lateral membrane domain is in contact with neighbouring cells, and the basal membrane domain adheres to the extracellular matrix (ECM) (Yeaman *et al.*, 1999). The formation of biological tubes is intimately linked to the development of apico-basal membrane polarity (Zegers *et al.*, 2003; Martin-Belmonte *et al.*, 2008; Lampugnani *et al.*, 2010), and a loss of cell polarity is frequently associated with defects in organ formation or with cancer development (McCaffrey and Macara, 2011; Rodriguez-Boulan and Macara, 2014).

In epithelial cells, apico-basal polarity is regulated by a conserved set of polarity protein complexes. These include the Par3 - aPKC - Par6 complex (Par complex), the CRB3 - Pals1 - PATJ complex (CRB3 complex), and the Scribble - Lgl - Dlg complex (Scrb complex) (Macara, 2004). The Par complex and the CRB3 complex localize to the apical region of the intercellular junctions which harbors the tight junctions (TJ) in vertebrates, whereas the Scrb complex localizes to the basolateral membrane domain (Macara, 2004). The Par complex and the CRB3 complex are biochemically linked through a direct association between Par6 and Pals1 (Hurd *et al.*, 2003; Wang *et al.*, 2004).

The endothelium represents a simple squamous epithelium that lines the surface of blood and lymphatic vessels. Endothelial cells are polarized and are connected by intercellular junctions which contain TJs and adherens junctions (AJs) (Dejana, 2004). As opposed to epithelial cell-cell junctions where the TJs are concentrated at the apical region of the junction and separated from the AJs, the TJs of endothelial cells are localized along the entire lateral junctional cleft and are intermingled with AJs and gap junctions (Simionescu *et*

al., 1975, 1976). Accordingly, proteins which in epithelial cells are separated by their specific localization in TJs and AJs including Occludin, ZO-1, or claudins, and cadherins or catenins, respectively, co-localize at endothelial cell-cell contacts (Schulze and Firth, 1993; Vorbrod and Dobrogowska, 2003; Ruffer *et al.*, 2004).

Despite a similar molecular composition of cell-cell junctions in epithelial cells and endothelial cells the mechanisms regulating cellular polarity and lumen formation are much less understood in endothelial cells. The major cell adhesion molecule at endothelial cell-cell junctions, vascular endothelial (VE)-cadherin directly interacts with components of the Par complex (Iden *et al.*, 2006; Lampugnani *et al.*, 2010; Tyler *et al.*, 2010) and regulates the localization of the Par complex regulator Tiam1 as well as the activity of aPKC at cell-cell junctions (Lampugnani *et al.*, 2002; Lampugnani *et al.*, 2010), suggesting a specific role in endothelial polarization. In the absence of VE-cadherin, endothelial cells fail to develop a polarized vascular lumen both in vitro and in vivo (Lampugnani *et al.*, 2010). The activity of VE-cadherin in regulating endothelial polarization and vascular lumen formation has been partially linked to its association with cerebral cavernous malformations (CCM) 1 / Krev interaction trapped (Krit) 1 protein (Lampugnani *et al.*, 2010). The physiological implications of its association with cell polarity proteins are not well understood.

Here, we describe an interaction of VE-cadherin with the polarity protein Pals1. Pals1 is a member of the membrane-associated guanylate kinase (MAGUK) family of scaffold proteins, which are characterized by a specific arrangement of protein-protein interaction domains including PSD-95/Dlg/ZO-1 (PDZ) domains, Src homology-3 (SH3) domains, Lin-2/Lin-7 (L27) domains, WW domains, and guanylate kinase (GK) domains (Funke *et al.*, 2005). In epithelial cells, Pals1 is localized at TJs where it links the integral membrane protein CRB3 to the multiple PDZ domain-containing scaffold protein Protein Associated with Tight Junctions (PATJ) to form the tripartite CRB3 - Pals1 - PATJ complex (Roh *et al.*, 2002; Makarova *et al.*, 2003). Pals1 also directly interacts with Par6 through a conserved motif that is not involved in the interactions with CRB3 and PATJ, and this association regulates the localization of these two polarity complexes at the TJs in a mutual way (Hurd *et al.*, 2003;

Wang *et al.*, 2004). A loss of Pals1 expression in MDCK cells results in delayed polarization, impaired barrier function and defective lumen formation (Roh *et al.*, 2003; Straight *et al.*, 2004). We find that VE-cadherin directly interacts with Pals1 and promotes the recruitment of Pals1 to intercellular junctions. Importantly, VE-cadherin mutants that cannot directly interact with Pals1 as well as VE-cadherin mutants that cannot directly interact with Par3 fail to support normal lumen development in three-dimensional culture. Our findings provide a molecular basis for the role of VE-cadherin during vascular lumen formation.

Results

The cell polarity protein Pals1 interacts with VE-cadherin in vascular endothelial cells

Given previous reports describing VE-cadherin as protein interacting with various polarity proteins including Par3 and Par6 (Iden *et al.*, 2006; Lampugnani *et al.*, 2010; Tyler *et al.*, 2010; Liu *et al.*, 2013) we tested if VE-cadherin associates with the cell polarity protein Pals1. We first analyzed the expression of Pals1 in endothelial cells by western blotting and immunoprecipitation (IP). We found that human umbilical vein endothelial cells (HUVEC) express two Pals1 isoforms corresponding to relative molecular masses (M_r) of approx. 82 kDa and 72 kDa (Figure 1A), which is similar to what has been found in epithelial cells (Straight *et al.*, 2004; Cao *et al.*, 2005). We next addressed the interaction between VE-cadherin and Pals1 by Co-IP experiments. VE-cadherin immunoprecipitates obtained from HUVECs contained the 82 kDa Pals1 isoform that is considered the canonical Pals1 isoform (<http://www.uniprot.org/uniprot/Q8N3R9>) (Figure 1B). In addition, Pals1 immunoprecipitates obtained from transfected HEK293T cells contained VE-cadherin (Figure 1C). To further corroborate these findings, we analyzed the expression and localization of Pals1 in cultured cells and in endothelial tissues. Pals1 co-localized with VE-cadherin at intercellular junctions of cultured HUVEC (Figure 1D), and it co-localized with VE-cadherin at cell-cell junctions after ectopic expression in HEK293T cells (Figure 1E). In addition, Pals1 was co-expressed with VE-cadherin in endothelial cells of the vascular plexus in the mouse retina at postnatal

day 6, as revealed by whole-mount staining of the retinal vasculature (Figure 1F). Together, these findings indicate that Pals1 exists in a complex with VE-cadherin that localizes at endothelial cell-cell contacts.

To analyze if the interaction between VE-cadherin and Pals1 is direct we performed GST-pulldown experiments using recombinant GST-fusion proteins encoding the cytoplasmic tail of VE-cadherin which were incubated with $^{35}\text{[S]}$ -methionine-labeled Pals1 generated in vitro using a coupled transcription/translation reticulocyte lysate system. The GST-VE-cadherin fusion protein precipitated Pals1 from reticulocyte lysates indicating that the interaction between VE-cadherin is direct (Figure 1G). GST-fusion proteins containing the cytoplasmic domains of E-cadherin or N-cadherin did not precipitate Pals1 in the same system indicating that Pals1 interacts specifically with VE-cadherin (Figure 1G). The cytoplasmic domain of VE-cadherin consists of three major regions, a juxtamembrane domain (JMD) which contains the binding site for p120ctn, a middle region, and a C-terminal catenin binding domain (CBD) which interacts with β - and γ -catenin (Vincent *et al.*, 2004). The JMD and the CBD are conserved among classical cadherins whereas the intervening region is less well conserved (Suzuki *et al.*, 1991; Tanihara *et al.*, 1994). We have previously found that Par3 interacts with the C-terminal PDZ domain binding motif of VE-cadherin (-EELII-COOH) whereas Par6 interacts with the JMD region through a site that is distinct from the p120ctn binding site (Iden *et al.*, 2006). To identify the binding site of Pals1 in VE-cadherin we performed GST-pulldown experiments with GST-VE-cadherin fusion proteins encompassing either the JMD and part of the intervening region, the intervening region, or the CBD of the cytoplasmic domain of VE-cadherin. Pals1 interacted with the VE-cadherin construct representing the JMD and part of the intervening region but not with constructs representing the intervening sequence alone or the CBD (Figure 1I, top panel). To further characterize the binding site in VE-cadherin, we introduced triple alanine mutations in the region flanking the p120ctn binding site within the JMD of VE-cadherin. Mutating amino acids located C-terminally of the p120ctn binding region partially reduced but did not abolish Pals1 binding (Figure 1I, bottom panel). On the other hand, mutating the amino acids located N-

terminally of the p120ctn binding region abolished the interaction. The interaction of VE-cadherin with p120ctn was not affected by these mutations (Figure 1I, bottom panel), suggesting that Pals1 and p120ctn do not compete for VE-cadherin binding. These observations indicate that Pals1 binding to VE-cadherin requires a short motif (R₆₂₁RRIRQ₆₂₆ in mouse VE-cadherin, R₆₂₁RRLRK₆₂₆ in human VE-cadherin) located N-terminally of the p120ctn binding site within the JMD of VE-cadherin.

VE-cadherin recruits Pals1 to cell-cell contacts

To address the question if VE-cadherin promotes the localization of Pals1 at cell-cell contacts, we analyzed the localization of Pals1 in HEK293T cells which have a fibroblastic morphology (Dupre-Crochet *et al.*, 2007). When transfected alone, Pals1 was localized predominantly in the cytoplasm and only very weakly at cell-cell junctions as indicated by a diffuse GFP signal throughout the cytoplasm and a weak GFP signal at cell boundaries (Figure 2A). When VE-cadherin was co-transfected with Pals1, the diffuse distribution of Pals1 in the cytoplasm was abolished and its junctional localization was strongly increased (Figure 2A) indicating that VE-cadherin promotes the localization of Pals1 at intercellular junctions. When we co-transfected Pals1 with the Pals1 binding mutant of VE-cadherin (VE-cad_3A4), we observed enrichment of Pals1 at intercellular junctions which was similar to that seen in cells co-transfected with wildtype VE-cadherin (Fig 2A, B). When Pals1 was co-transfected with the Par3 binding mutant of VE-cadherin (VE-cad_Δ5), Pals1 was not enriched at cell-cell junctions but localized predominantly in the cytoplasm, similar to cells transfected with Pals1 alone (Figure 2, A and B). These observations indicate that VE-cadherin promotes Pals1 localization at intercellular junctions, and that this activity is not mediated through the membrane-proximal motif involved in direct Pals1 binding but through the PDZ domain binding motif at the C-terminus of VE-cadherin involved in Par3 binding (Iden *et al.*, 2006; Tyler *et al.*, 2010). To test if Par3 mediates Pals1 binding to VE-cadherin, we co-expressed VE-cadherin and Pals1 in Par3-depleted HEK293T cells. The absence of Par3 did not impair the recruitment of Pals1 by VE-cadherin to cell-cell contacts, excluding

Par3 as mediator of the interaction between Pals1 and VE-cadherin (Suppl. Figure 1). To address the question if VE-cadherin is required for the localization of Pals1 at intercellular junctions of endothelial cells, we analyzed the subcellular localization of Pals1 in endothelial cells depleted of VE-cadherin. In control siRNA-transfected cells Pals1 appeared as thin signal lining the intercellular junctions (Figure 2C). In VE-cadherin knockdown cells Pals1 was reduced but still detectable at intercellular junctions, despite a complete knockdown of VE-cadherin (Figure 2C, bottom panels). These observations thus indicate that VE-cadherin is not mandatory for Pals1 localization at intercellular junctions. Most likely, the presence of Pals1 at cell-cell junctions of VE-cadherin-depleted endothelial cells is due to the ability of Pals1 to interact with multiple binding partners at cell-cell junctions including the cell polarity proteins CRB3, PATJ, and Par6 (Roh *et al.*, 2002; Hurd *et al.*, 2003; Makarova *et al.*, 2003; Wang *et al.*, 2004; Michel *et al.*, 2005).

VE-cadherin regulates vascular lumen formation through Pals1 and Par3 binding

To address the functional relevance of the direct interactions between VE-cadherin and the two polarity proteins Pals1 and Par3, we replaced endogenous VE-cadherin in HUVECs with mutants of VE-cadherin that have lost the ability to directly interact with Pals1 or Par3. HUVECs were transduced simultaneously with virus particles encoding a shRNA directed against human VE-cadherin and virus particles encoding shRNA-insensitive murine VE-cadherin cDNA constructs containing mutations (VE-cad_3A4) or deletions (VE-cad_Δ5) in the binding regions for Pals1 or Par3, respectively. We first analyzed the knockdown efficiencies of endogenous VE-cadherin as well as the levels of expression and subcellular localizations of the ectopic murine VE-cadherin constructs by western blot analyses and indirect immunofluorescence. Endogenous VE-cadherin was efficiently depleted in all cell populations generated (Figure 3, A and B). The overall expression levels of the ectopic VE-cadherin constructs were similar in all cell populations with slightly higher levels of the VE-cad_3A4 and VE-cad_Δ5 constructs as compared to the VE-cad_FL and VE-cad/3A4_Δ5 constructs (Figure 3A). All VE-cadherin constructs were specifically localized at

interendothelial junctions (Figure 3B). In addition, the Pals-1 and Par3 binding mutant showed the same subcellular localization like VE-cadherin WT when ectopically expressed in highly polarizing MDCK cells grown on polycarbonate filters, i.e. exclusive localization at intercellular junctions (Suppl. Fig 2). Finally, the mutations did not impair the adhesive activity of VE-cadherin as analyzed by aggregation assays of transfected CHO cells (Suppl. Figure 3). These findings indicated that the introduced mutations did neither affect the subcellular localization of VE-cadherin nor change its adhesive activity.

We then analyzed lumen formation in a three-dimensional (3D) collagen gel assay which mimicks the process of vasculogenesis in vitro (Koh *et al.*, 2008). Lentivirally transduced HUVECs were mixed with collagen type I at 4°C, then transferred to 37°C to allow solidification of the collagen, and subsequently grown for 48 h. Phase contrast images indicated that HUVECs developed lumen-containing spheroids under these conditions (Figure 4A, left panel). Lumen formation was confirmed by 3D reconstruction of confocal sections (Suppl. Movie 1). We next analyzed spheroid and lumen size by using the GFP fluorescence signal resulting from the lentiviral transduction (Figure 4A, right panel). Cross-sections of spheroids were used to analyze the spheroid and lumen size. Control cells developed into spheroids that commonly consisted of three or more cells with a cross-sectional area of approx. 3200 μm^2 and a cross-sectional lumen area of approx. 1700 μm^2 (Figure 4, B and C). Confocal microscopy analysis indicated that the cells developed 3D-spheroids consisting of a single lumen that was surrounded by two to three endothelial cells with a thin cytoplasm (Figure 4B), which is similar to what has been observed previously (Davis and Camarillo, 1996; Lampugnani *et al.*, 2010). VE-cadherin depletion resulted in a dramatic reduction of cross-sectional spheroid and lumen size. In addition, the cells frequently contained intracellular vacuoles indicative for early stages of lumen formation (Kamei *et al.*, 2006; Lampugnani *et al.*, 2010; Wang *et al.*, 2010) (Figure 4B, VE-cad shRNA). Ectopic expression of mVE-cadherin in VE-cadherin-depleted HUVECs cells restored spheroid and lumen size and also the ability of cells to develop multicellular spheroids with a single lumen (Figure 4, B and C, mVE-cad_FL). Interestingly, ectopic

expression of the Pals1 binding mutant of VE-cadherin resulted in spheroids which were similar to spheroids originating from VE-cadherin-depleted cells. Both cross-sectional spheroid and lumen areas were not significantly larger than in VE-cadherin knockdown spheroids (Figure 4, B and C, VE-cad_3A4). In addition, instead of a single lumen the spheroids frequently contained multiple small lumens or vacuoles, and the cytoplasm of the cells was enlarged when compared with the thin cytoplasm of mVE-cad_FL-expressing VE-cadherin knockdown endothelial cells (Figure 4, B and C, compare VE-cad_FL and VE-cad_3A4). Similar to the Pals1 binding mutant the Par3 binding mutant of VE-cadherin was unable to re-establish cross-sectional spheroid and lumen areas, and the spheroids contained intracellular vacuoles instead of a single lumen (Figure 4, B and C, VE-cad_Δ5). Ectopic expression of a VE-cadherin mutant deficient for both Pals1 and Par3 binding resulted in spheroids that resembled those obtained after re-expression of the individual binding mutants alone, i.e. spheroids with reduced cross-sectional spheroid and lumen areas and with either multiple lumens or vacuoles surrounded by cells with highly enlarged cytoplasm (Figure 4, B and C, VE-cad_3A4Δ5). The effects of the different VE-cadherin mutant constructs on spheroid size and lumen size are quantified in Figure 4C. Together, these findings indicate that both the Pals1 binding motif in the JMD of VE-cadherin as well as the Par3 binding motif at the C-terminus of the CBD of VE-cadherin are required for normal spheroid development and lumen formation.

To directly analyze Pals1 function in spheroid development and lumen formation, we performed spheroid assays with Pals1-depleted HUVECs. Surprisingly, the loss of Pals1 expression resulted in a drastically reduced number of spheroids (Suppl. Figure 4) suggesting a potential role of Pals1 in endothelial cell proliferation or survival, which has been observed before in neural progenitor cells (Kim *et al.*, 2010; Park *et al.*, 2016). Spheroids that developed from Pals1 knockdown cells had significantly smaller lumens than control cells (Figure 4D, Suppl. Figure 4), which is similar to what we observed in VE-cadherin knockdown cysts. These findings indicate that Pals1 regulates spheroid

development and suggest that this function is mediated through different mechanisms including cell proliferation, cell survival and cell polarization.

VE-cadherin regulates endothelial membrane polarity through Pals1 and Par3

We next analyzed membrane polarity in spheroids originating from either control HUVECs, VE-cadherin knockdown HUVECs, or HUVECs with endogenous VE-cadherin replaced by Pals1- or Par3- or Pals1/Par3 binding mutants of VE-cadherin. Control cells developed regular spheroids with a central lumen, in which the apical membrane marker podocalyxin (Podxl) was enriched along the apical membrane above the nucleus (Figure 5). The basal marker Collagen IV (Coll IV) was restricted to the basal surface of the cells where it was evenly distributed, which is similar to what has been observed before (Lampugnani *et al.*, 2010). Depletion of VE-cadherin resulted in much smaller spheroids in which the polar distribution of Podxl and Coll IV was disturbed (Figure 5). Podxl was enriched at specific sites along intracellular vacuoles and absent at others. Similarly, Coll IV was unevenly distributed along the basal membrane domain. Ectopic expression of murine VE-cadherin completely restored this polarity defect induced by depletion of endogenous VE-cadherin. Importantly, neither the Pals-1 binding mutant nor the Par3 binding mutant of VE-cadherin restored Podxl membrane polarity when expressed in VE-cadherin-depleted endothelial cells. In both VE-cadherin_{3A4}- and VE-cadherin_{Δ5}-reconstituted cells Podxl was enriched at specific sites within vacuoles (Figure 5). The localization of Coll IV was less severely affected than Podxl as the protein was still restricted to the basal membrane domain facing the collagen matrix. The phenotype of the VE-cadherin_{3A4Δ5}-reconstituted spheroids was similar to the phenotypes seen for cells expressing the single mutants, i.e. ectopic localization of Podxl at intracellular vacuoles but mostly basal localization of Coll IV (Figure 5). Together, these observations indicate that VE-cadherin regulates apical membrane specification and vascular lumen formation through its interaction with both Pals1 and Par3.

Discussion

In this study, we identify the conserved cell polarity protein Pals1 as a novel binding partner of VE-cadherin. The interaction is direct and involves a membrane-proximal region in the cytoplasmic domain of VE-cadherin. We find that both the direct interaction with Pals1 and the previously identified interaction with Par3 are critical for the ability of VE-cadherin to regulate apico-basal polarity and vascular lumen formation. Our study identifies the two conserved cell polarity proteins Pals1 and Par3 as mediators of VE-cadherin-regulated polarity in vascular endothelial cells.

Interestingly, although VE-cadherin serves as an interaction partner of Pals1, we found that both after depletion of VE-cadherin in endothelial cells and upon ectopic expression of the Pals1 binding mutant of VE-cadherin in HEK293T cells, Pals1 can still localize at cell-cell contacts. The cell-cell contact localization in VE-cadherin knockdown endothelial cells can most likely be explained by the multiple protein interactions that have been described for Pals1. In epithelial cells Pals1 is part of the CRB3 - Pals1 - PATJ complex and interacts directly with both CRB3 and PATJ (Roh *et al.*, 2002; Michel *et al.*, 2005) which provides one possible mechanism for Pals1 localization in the absence of VE-cadherin. Furthermore, PATJ can interact with other transmembrane proteins including JAM-A and Nectin-1 (Adachi *et al.*, 2009), providing a second possible mechanism for Pals1 localization in the absence of VE-cadherin. The normal recruitment of Pals1 in HEK293T cells expressing the VE-cadherin/3A4 mutant but not with the VE-cadherin PDZ domain binding mutant (VE-cadherin/ Δ 5) could also be explained by an indirect association of Pals1 with VE-cadherin through a PDZ domain protein interacting with VE-cadherin. Par3 would be likely candidate since it can interact with the Pals1 binding partner Par6 (Hurd *et al.*, 2003; Wang *et al.*, 2004). However, we can rule out Par3 as mediator of this interaction since the absence of Par3 did not affect Pals1 recruitment in transfected HEK293T cells (Suppl. Figure 1).

Our studies further emphasize an important role for VE-cadherin in vascular lumen formation and identify two molecular binding partners through which VE-cadherin mediates

this activity, i.e. Pals1 and Par3. According to the current model, de novo lumen formation is initiated when two apposing cells in endothelial cell cords form close cell-cell contacts via VE-cadherin trans-homophilic interactions (Zeeb *et al.*, 2010). VE-cadherin recruits the sialomucins CD34 and Podxl to the site of cell-cell contact thereby initiating the separation of the two contacting cell membranes by way of repulsive forces mediated by the negatively charged carbohydrates on sialomucins (Strilic *et al.*, 2009; Strilic *et al.*, 2010). The localization of CD34 and Podxl at the cell-cell contact site is followed by the recruitment of Moesin which stabilizes F-actin at the newly formed apical membrane. VEGF signaling triggers the interaction of non muscle (nm) Myosin II with F-actin and acto-myosin-driven cell shape changes that are required for lumen formation (Zeeb *et al.*, 2010). The further steps following these primary events are less well understood. The initiation of apical membrane specification driven by VE-cadherin-mediated cell adhesion must be reinforced and propagated in order to maintain apico-basal membrane polarity, as described in epithelial cells (Yeaman *et al.*, 1999). We hypothesize that VE-cadherin contributes to the maintenance of apico-basal membrane polarity and stable lumen formation via both polarity proteins, Pals1 and Par3.

The interaction with Pals1 probably serves to stabilize F-actin at the apical membrane. In parietal epithelial cells, Pals1 interacts with Ezrin, a member of the Ezrin - Radixin - Moesin family, and regulates Ezrin recruitment to the apical membrane domain, which is required for the remodeling of the apical membrane cytoskeleton associated with vectorial secretion (Cao *et al.*, 2005). In MDCK epithelial cells, a complex consisting of Podxl, NHERF1 and Ezrin promotes apical membrane identity, most likely through the activity of phosphorylated ezrin to stabilize the localization of a NHERF1 - Podxl complex at the apical membrane (Bryant *et al.*, 2014). The ERM family protein predominantly expressed in endothelial cells is Moesin (Fehon *et al.*, 2010). Thus, by recruiting Moesin through Pals1 to cell-cell junctions VE-cadherin most likely contributes to the formation of the apical membrane. In agreement with this, VE-cadherin and Moesin1 are required for the establishment and maintenance of apico-basal polarity during lumen formation in the intersegmental vessels of the zebrafish embryo

(Wang *et al.*, 2010). Of note, Pals1 has recently been shown to be involved in the regulation of cell cycle progression and cell survival of neural progenitor cells in the cerebrum and in the cerebellum which is in part mediated by an influence on the mammalian target of rapamycin (mTOR) signaling pathway (Kim *et al.*, 2010; Park *et al.*, 2016). Our observations of a strongly reduced number of spheroids after Pals1 depletion in HUVECs (Suppl. Figure 4) are in line with a similar role of Pals1 in endothelial cells. Thus, we speculate that Pals1 contributes to vascular lumen formation not only by regulating apical membrane identity but also by maintaining endothelial cells in a proliferative and anti-apoptotic state. The impaired spheroid formation in cells expressing the Pals1 binding-defective mutant of VE-cadherin (Figure 4B, C) could then in part be explained by a putative mislocalization of Pals1 resulting in altered or defective mTOR signaling and reduced cell survival.

The interaction of VE-cadherin with Par3 probably contributes to the development of apical membrane identity. Par3 interacts with various phosphoinositides including PtdIns(4,5)P2 and PtdIns(3,4,5)P3 and with the lipid phosphatase Phosphatase and Tensin Homolog (PTEN) (Wu *et al.*, 2007). In polarized epithelial cells, PtdIns(4,5)P2 is localized at the apical membrane whereas PtdIns(3,4,5)P3 is localized at the basolateral membrane but excluded from the apical membrane (Gassama-Diagne *et al.*, 2006). The apical segregation of phosphoinositides is regulated by apically localized PTEN (Martin-Belmonte *et al.*, 2007), which confers apical membrane identity by dephosphorylating apical PtdIns(3,4,5)P3 to PtdIns(4,5)P2. Intriguingly, loss of PTEN function in MDCK cells as well as loss of apical localization of a Par3 - aPKC complex during mouse mammary epithelium development inhibits lumen formation and ductal morphogenesis, respectively (Martin-Belmonte *et al.*, 2007; Elias *et al.*, 2015). Thus, by recruiting PTEN through Par3, VE-cadherin probably contributes to the specification of apical and basolateral membranes as a prerequisite for lumen formation.

One important observation of our study is that the lack of direct binding of Pals1 and Par3 to VE-cadherin results in a phenotype comparable to the complete lack of VE-cadherin expression, i.e. disorganized spheroids which contain one or several large vacuoles (Figs 4,

5). The formation of the vascular lumen has been proposed to be regulated either by fusion of intracellular vesicles and vacuoles which coalesce with vacuoles from neighbouring cells (cell hollowing) or by the selective recruitment of apical membrane determinants to sites of cell-cell contacts at early stages of lumenization followed by membrane separation and cell-cell contact segregation (cord hollowing) (Iruela-Arispe and Davis, 2009; Neufeld *et al.*, 2014). It is likely that both mechanisms contribute to tube formation, and the relative contributions of the two mechanisms might depend on the type of blood vessel and the vascular bed (Wang *et al.*, 2010). In addition, vacuoles do not only fuse and coalesce with vacuoles from adjacent cells, but can also fuse with the luminal membrane and thereby contribute to lumen expansion during the cord hollowing mechanism of lumenization (Wang *et al.*, 2010; Zovein *et al.*, 2010). Interestingly, the lack of $\beta 1$ integrin in nascent endothelial cells results in reduced expression of Par3 and mislocalization of VE-cadherin, concomitant with abnormal accumulation of intracellular vacuoles which most likely fail to fuse with the nascent apical membrane as a result of disturbed membrane polarity (Zovein *et al.*, 2010; Yamamoto *et al.*, 2015). Together with these published findings our observations suggest that VE-cadherin interacts with the two cell polarity regulators Pals1 and Par3 downstream of $\beta 1$ integrin to regulate apical membrane specification and luminal vesicle fusion during lumen morphogenesis.

Materials and Methods

Cell culture and transfections

Human umbilical vein endothelial cells (HUVECs) were obtained from PromoCell (PromoCell GmbH, Heidelberg, Germany; Cat. # C-12203) and maintained in Endothelial Growth Medium 2 (PromoCell, Cat. # C-22011) as recommended by the manufacturer. HEK293T and MDCKII cells (Sigma-Aldrich, München, Germany) were maintained in DMEM (Sigma-Aldrich, Munich, Germany) supplemented with 10 % fetal calf serum (FCS), 2 mM glutamine and 100 U/ml penicillin/streptomycin. VE-cadherin-transfected CHO cells (Iden *et al.*, 2006)

were maintained in α -MEM (Biochrom GmbH, Berlin, Germany) supplemented with 10 % FCS, 2 mM glutamine, 100 U/ml penicillin/streptomycin and 500 μ g/ml G418. Transient transfections of plasmids in HEK293T, MDCKII cells, and CHO cells were performed using either Xfect Transfection Reagent (Clontech Laboratories Inc., Mountain View, CA) or Lipofectamine® 2000 Transfection Reagent (Life Technologies GmbH, Darmstadt, Germany) according to the manufacturer's instructions. Short interfering RNA oligonucleotide (siRNA) transfections of HUVECs were performed using Xfect Transfection Reagent according to the manufacturer's instructions. Short hairpin RNA (shRNA)-encoding plasmids as well as cDNA plasmids encoding various murine VE-cadherin constructs were introduced into HUVECs by lentiviral transduction. Lentiviral particles were produced in HEK293T cells by cotransfection of the lentiviral vectors pLVTHM (Addgene plasmid #12247) or pCDH-CMV-MCS-EF1-Puro (System Biosciences, Mountain View, CA) with the packaging vectors psPAX2 (Addgene plasmid #12260) and pMD2.G (Addgene plasmid #12259) in a ratio of 4:3:1. The following day HEK293T culture medium was replaced by Endothelial Growth Medium 2, and after 24 hours of incubation lentivirus particle-containing HEK293T supernatants were transferred to HUVECs. After overnight incubation with lentivirus particles, the cells were cultured for two additional days in the absence of virus particles prior to analysis.

Antibodies and reagents

The following antibodies were used: goat pAb anti-VE-cadherin (sc-6458, Santa Cruz Biotechnology, Dallas, TX), mouse mAb anti-VE-cadherin (610252, BD Biosciences, Heidelberg, Germany), rat mAb anti-VE-cadherin 11D4.1 (550548 BD Biosciences), mouse mAb anti-Flag-tag (F1804, Sigma-Aldrich), rabbit pAb anti-Flag-tag (F7425, Sigma-Aldrich), rabbit pAb anti-ZO1 (61-7300, Thermo Fisher Scientific, Waltham, MA), rabbit pAb anti-MPP5/Pals1 (HPA000993, Sigma-Aldrich), mouse mAb anti- α -Tubulin (T5168, Sigma-Aldrich), goat pAb anti-Podocalyxin (AF1658, R&D Systems, Minneapolis, MN), rabbit pAb anti-collagen IV (2150-0140, AbD Serotec, Puchheim, Germany), and rabbit pAb anti-Par3

(Iden *et al.*, 2006). The following reagents were used: basic fibroblast growth factor (bFGF, Peprtech, Hamburg, Germany), vascular endothelial growth factor (VEGF, Peprtech).

RNA interference

To deplete VE-cadherin in HUVECs, the following siRNA oligonucleotide was used: 5'-AGAUGCAGAGGCCUCAUGAUTT-3' (Schafer *et al.*, 2003). As control siRNA, a non-targeting siRNA (On-TARGETplus Non-targeting siRNA, Thermo Fisher Scientific) was used. For lentiviral downregulation of VE-cadherin in HUVEC the following oligonucleotides were annealed and cloned into the shRNA expression vector pLVTHM: 5'-CGCGTCCCCAGATGCAGAGGCTCATGATTTCAAGAGAATCATGAGCCTCTGCATCTTTT TTGAAAT-3', 5'-CGATTTCCAAAAAGATGCAGAGGCTCATGATTCTCTTGAATCATGAGCCTCTGCATCTGGGA-3'. As negative control, the two following oligonucleotides were annealed and cloned into pLVTHM: 5'-CGCGTCCCCGGCAGCACAAGTGCCTTGT CAAGAGACAAGTGCAGTTGTGCTGCCTTTTTGGAAAT-3' and 5'-CGATTTCCAAAAAGG CAGCACAAGTGCCTTGTCTCTTGAACAAGTGCAGTTGTGCTGCCGGGA-3'. These two oligos encode for a shRNA not targeting human VE-cadherin. Knockdown of Pals1 was performed using a commercially available pool of three Pals1-specific shRNAs-encoding lentiviral vector plasmids (Santa Cruz, sc-43991-SH). Knockdown of Par3 was performed using a lentiviral shRNA plasmid targeting human PARD3 (Dharmacon, RHS3979-201817648).

DNA constructs

The following GST-fusion constructs were described previously: murine VE-cadherin cytoplasmic domain lacking five AA at the C-terminal end (VE-cad/CP Δ 5, AA621-784), murine E-cadherin cytoplasmic domain (E-cad/CP, AA732-882), murine N-cadherin cytoplasmic domain (N-cad/CP (AA747-906) (Iden *et al.*, 2006). The following GST fusion proteins which contain specific subregions or mutations of the cytoplasmic domain of murine VE-cadherin were generated in pGEX-4T-1: VE-cad/proximal (AA621-689), VE-cad/middle

(AA661-730), VE-cad/distal (AA714-784), VE-cad/3A1 (AA621-689, AA667-669 replaced by Ala), VE-cad/3A2 (AA621-689, AA670-672 replaced by Ala), VE-cad/3A3 (AA621-689, AA673-675 replaced by Ala), VE-cad/3A4 (AA621-689, AA621-623 replaced by Ala), (VE-cad/3A5 AA621-689, AA624-626 replaced by Ala). The following expression constructs were cloned into pcDNA3 and pCDH-CMV-MCS-EF1-Puro: mVE-cad_FL (murine full length VE-cadherin), mVE-cad_3A4 (AA621-623 replaced by Ala), mVE-cad_Δ5 (AA780-784 deleted), mVE-cad_3A4Δ5 (AA621-623 replaced by Ala and AA780-784 deleted). Human Pals1 full length cDNA was cloned into pcDNA3.1/nV5-Dest plasmid vector (Invitrogen, Carlsbad, CA) for in vitro transcription/translation, into pcDNA-DEST53 (Invitrogen) for ectopic expression of N-terminally GFP-tagged Pals1, and into pQCXIP-3xFlag for ectopic expression of N-terminally Flag-tagged Pals1.

Immunoprecipitation and western blot analysis

For immunoprecipitations cells were lysed in lysis buffer [50 mM Tris HCl (pH 7.4), 1% (v/v) Nonidet P-40 (AppliChem GmbH, Darmstadt, Germany), 150 mM NaCl, protease inhibitors (Protease Inhibitor Cocktail tablets "Complete", Roche)] for 30 min on ice, and then centrifuged at 4°C. Postnuclear supernatants were incubated with 3 µg of antibodies coupled to Protein A- or Protein G-sepharose beads (GE-Healthcare) overnight at 4°C. Immune complex-captured beads were washed five times with lysis buffer without inhibitors and boiled in SDS sample buffer containing 0.1 M DTT. The proteins were separated by SDS-PAGE and analyzed by western blotting with near-infrared fluorescence detection (Odyssey imaging system and IRDye 800CW conjugated antibodies, LI-COR Biosciences GmbH, Bad Homburg, Germany).

In vitro binding experiments

In vitro binding experiments were performed as described previously (Ebnet *et al.*, 2000). Briefly, GST fusion proteins were expressed in E.coli BL21 (GE Healthcare, Solingen, Germany). Bacteria were lysed by passaging through a French pressure cell and GST-fusion

proteins were purified by affinity chromatography. Protein solutions were adjusted to 50% (w/v) glycerol and stored at -20°C. For GST pulldown experiments the prey proteins were generated in vitro using the TNT T7-coupled reticulocyte lysate system (Promega Corp., Madison, WI) in the presence of ³⁵[S]-labeled methionine as described by the manufacturer. 10 µl of the translation reactions were incubated with 3 mg GST fusion proteins immobilized on glutathione-Sepharose 4B beads (Life Technologies) for 2 h at 4°C under constant agitation. Beads were washed five times with buffer B (10 mM Hepes pH 7.2, 100 mM KCl, 1 mM MgCl₂, 0.1% Triton X-100). Bound proteins were eluted by boiling for 5 min in SDS sample buffer, subjected to SDS-PAGE and analyzed by fluorography.

Immunofluorescence microscopy

Immunofluorescence analyses were performed with cells grown on fibronectin-coated chamber slides (Lab-Tek II, Thermo Fisher Scientific). Cells were fixed in either 4 % paraformaldehyde for 10 min or in ice-cold MeOH for 5 min. Paraformaldehyde-fixed cells were permeabilized for 10 min in PBS containing 0.5% Triton X-100 and subsequently washed with PBS containing 100 mM glycine for 10 min. Blocking was performed for 1 h at room temperature with blocking buffer (PBS, 10% FCS, 0.2% Triton X-100, 0.05% Tween 20, 0.02% BSA) followed by incubation with primary antibodies in blocking buffer overnight at 4 °C. Incubation with secondary antibodies (AlexaFluor488, AlexaFluor594 and AlexaFluor647-conjugated, highly cross-adsorbed secondary antibodies, Life Technologies) and 1 µg/mL DAPI (Sigma-Aldrich) was performed in blocking buffer for 1 h at RT. Finally samples were washed with blocking buffer and mounted in fluorescence mounting medium (Mowiol 4-88, Sigma-Aldrich). Immunofluorescence microscopy was performed using the confocal microscope LSM 780 (Carl Zeiss, Jena, Germany) equipped with the objective lenses Plan-Neofluar 20x/0.5 and Plan-Apochromat × 63/1.4 oil differential interference contrast (Carl Zeiss). Phase contrast microscopy was performed using an EVOS Fluorescence Microscope (Advanced Microscopy Group, Mill Creek, WA). To quantify Pals1 localization in transfected HEK293T cells, the cell-cell contact area was defined as the area

reaching 0.25 μm into each of the two contacting cells. The Pals1 intensity is given as ratio of the mean intensities measured at cell-cell contacts and measured in the cytoplasm of the contacting cells.

3D culture

HUVEC 3D cultures in collagen gels were performed as described (Bayless and Davis, 2002; Lampugnani *et al.*, 2010). Briefly, HUVECs were seeded at a concentration of 5×10^5 cells/ml in 3.5 mg/ml collagen gels (collagen type I from rat tail, high concentration, BD Biosciences). For confocal immunofluorescence analysis 200 μl of the cell suspension in collagen was added to each well of a μ -Slide 8 Well (ibidi GmbH, Martinsried, Germany). After incubation for 30 min at 37°C in a humidified CO₂ incubator the collagen gel was overlaid with 200 μl medium (M199 with 1% FCS, insulin-transferrin-selenium supplement, 50 ng/ml PMA, 50 $\mu\text{g}/\text{ml}$ ascorbic acid, 40 ng/ml VEGF, 40 ng/ml bFGF). HUVEC 3D cultures were cultivated for 48 h, then fixed and processed for immunofluorescence microscopy. Spheroids were fixed in 4 % paraformaldehyde for 35 min and permeabilized in PBS containing 0.5% Triton X-100 for 20 min. Incubation with primary and secondary antibodies was performed overnight. Immunostained spheroids were analyzed by confocal microscopy as described above. For spheroid and lumen size quantitation, 50 μl of the cell suspension in collagen was added per well of a 96 well microplate. Spheroid and lumen size was analyzed by taking cross-sectional images of randomly selected spheroids at 20x magnification. Areas were measured with ImageJ software (NIH, Bethesda, MD).

Cell aggregation assays

For aggregation assays, CHO cells transfected with VE-cadherin constructs were harvested, washed in HBSS, 2 mM CaCl₂, 25 mM HEPES, then counted and adjusted to $1 \times 10^6/\text{ml}$. 3 ml of cell suspension were added to a 30-mm BSA-coated petri dish and incubated on a horizontal shaker at 35 rpm for 45 min at 37°C. Cells were fixed by addition of 500 μl 25%

glutaraldehyde and incubation on ice for 30 min. The number of particles was counted using ImageJ software. Particles larger than $256 \mu\text{m}^2$ were considered as aggregates.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6.0 (GraphPad Software, La Jolla, CA). Statistical significances in Figure 2B and Figure 4C were calculated by one-way ANOVA with Dunnett's post hoc test. Statistical significances in Supplemental Figures 1 and 3 was calculated by one-way ANOVA with Dunnett's post hoc test.

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Authorship and conflict of interest statements

Author contributions: B.F.B. and K.E. designed and conceived the study. B.F.B. performed most of the experimental work. T.S., C.H., D.K., D.P. and F.M. performed experiments. T.W. provided important reagents. B.F.B., M.N., V.G. and K.E. analyzed the data. B.F.B. and K.E. wrote the manuscript. The authors declare that they have no conflict of interest.

References

Adachi, M., Hamazaki, Y., Kobayashi, Y., Itoh, M., Tsukita, S., Furuse, M., and Tsukita, S. (2009). Similar and distinct properties of MUPP1 and Patj, two homologous PDZ domain-containing tight-junction proteins. *Mol Cell Biol* 29, 2372-2389.

Bayless, K.J., and Davis, G.E. (2002). The Cdc42 and Rac1 GTPases are required for capillary lumen formation in three-dimensional extracellular matrices. *J Cell Sci* 115, 1123-1136.

- Bryant, D.M., Roignot, J., Datta, A., Overeem, A.W., Kim, M., Yu, W., Peng, X., Eastburn, D.J., Ewald, A.J., Werb, Z., and Mostov, K.E. (2014). A molecular switch for the orientation of epithelial cell polarization. *Developmental cell* 31, 171-187.
- Cao, X., Ding, X., Guo, Z., Zhou, R., Wang, F., Long, F., Wu, F., Bi, F., Wang, Q., Fan, D., Forte, J.G., Teng, M., and Yao, X. (2005). PALS1 specifies the localization of ezrin to the apical membrane of gastric parietal cells. *The Journal of biological chemistry* 280, 13584-13592.
- Davis, G.E., and Camarillo, C.W. (1996). An alpha 2 beta 1 integrin-dependent pinocytic mechanism involving intracellular vacuole formation and coalescence regulates capillary lumen and tube formation in three-dimensional collagen matrix. *Experimental cell research* 224, 39-51.
- Dejana, E. (2004). Endothelial cell-cell junctions: happy together. *Nat Rev Mol Cell Biol* 5, 261-270.
- Dupre-Crochet, S., Figueroa, A., Hogan, C., Ferber, E.C., Bialucha, C.U., Adams, J., Richardson, E.C., and Fujita, Y. (2007). Casein kinase 1 is a novel negative regulator of E-cadherin-based cell-cell contacts. *Molecular and cellular biology* 27, 3804-3816.
- Ebnet, K., Schulz, C.U., Meyer Zu Brickwedde, M.K., Pendl, G.G., and Vestweber, D. (2000). Junctional adhesion molecule interacts with the PDZ domain-containing proteins AF-6 and ZO-1. *J Biol Chem* 275, 27979-27988.
- Elias, S., McGuire, J.R., Yu, H., and Humbert, S. (2015). Huntingtin Is Required for Epithelial Polarity through RAB11A-Mediated Apical Trafficking of PAR3-aPKC. *PLoS biology* 13, e1002142.
- Fehon, R.G., McClatchey, A.I., and Bretscher, A. (2010). Organizing the cell cortex: the role of ERM proteins. *Nature reviews. Molecular cell biology* 11, 276-287.
- Funke, L., Dakoji, S., and Brettt, D.S. (2005). Membrane-associated guanylate kinases regulate adhesion and plasticity at cell junctions. *Annu Rev Biochem* 74, 219-245.
- Gassama-Diagne, A., Yu, W., ter Beest, M., Martin-Belmonte, F., Kierbel, A., Engel, J., and Mostov, K. (2006). Phosphatidylinositol-3,4,5-trisphosphate regulates the formation of the basolateral plasma membrane in epithelial cells. *Nature cell biology* 8, 963-970.
- Hurd, T.W., Gao, L., Roh, M.H., Macara, I.G., and Margolis, B. (2003). Direct interaction of two polarity complexes implicated in epithelial tight junction assembly. *Nat Cell Biol* 5, 137-142.
- Iden, S., Rehder, D., August, B., Suzuki, A., Wolburg-Buchholz, K., Wolburg, H., Ohno, S., Behrens, J., Vestweber, D., and Ebnet, K. (2006). A distinct PAR complex associates physically with VE-cadherin in vertebrate endothelial cells. *EMBO Rep* 7, 1239-1246.
- Iruela-Arispe, M.L., and Davis, G.E. (2009). Cellular and molecular mechanisms of vascular lumen formation. *Dev Cell* 16, 222-231.
- Kamei, M., Saunders, W.B., Bayless, K.J., Dye, L., Davis, G.E., and Weinstein, B.M. (2006). Endothelial tubes assemble from intracellular vacuoles in vivo. *Nature* 442, 453-456.

- Kim, S., Lehtinen, M.K., Sessa, A., Zappaterra, M.W., Cho, S.H., Gonzalez, D., Boggan, B., Austin, C.A., Wijnholds, J., Gambello, M.J., Malicki, J., LaMantia, A.S., Broccoli, V., and Walsh, C.A. (2010). The apical complex couples cell fate and cell survival to cerebral cortical development. *Neuron* 66, 69-84.
- Koh, W., Stratman, A.N., Sacharidou, A., and Davis, G.E. (2008). In vitro three dimensional collagen matrix models of endothelial lumen formation during vasculogenesis and angiogenesis. *Methods in enzymology* 443, 83-101.
- Lampugnani, M.G., Orsenigo, F., Rudini, N., Maddaluno, L., Boulday, G., Chapon, F., and Dejana, E. (2010). CCM1 regulates vascular-lumen organization by inducing endothelial polarity. *J Cell Sci* 123, 1073-1080.
- Lampugnani, M.G., Zanetti, A., Breviario, F., Balconi, G., Orsenigo, F., Corada, M., Spagnuolo, R., Betson, M., Braga, V., and Dejana, E. (2002). VE-cadherin regulates endothelial actin activating Rac and increasing membrane association of Tiam. *Molecular biology of the cell* 13, 1175-1189.
- Liu, Y., Collins, C., Kiosses, W.B., Murray, A.M., Joshi, M., Shepherd, T.R., Fuentes, E.J., and Tzima, E. (2013). A novel pathway spatiotemporally activates Rac1 and redox signaling in response to fluid shear stress. *The Journal of cell biology* 201, 863-873.
- Lubarsky, B., and Krasnow, M.A. (2003). Tube morphogenesis: making and shaping biological tubes. *Cell* 112, 19-28.
- Macara, I.G. (2004). Parsing the polarity code. *Nat Rev Mol Cell Biol* 5, 220-231.
- Makarova, O., Roh, M.H., Liu, C.J., Laurinec, S., and Margolis, B. (2003). Mammalian Crumbs3 is a small transmembrane protein linked to protein associated with Lin-7 (Pals1). *Gene* 302, 21-29.
- Martin-Belmonte, F., Gassama, A., Datta, A., Yu, W., Rescher, U., Gerke, V., and Mostov, K. (2007). PTEN-mediated apical segregation of phosphoinositides controls epithelial morphogenesis through Cdc42. *Cell* 128, 383-397.
- Martin-Belmonte, F., Yu, W., Rodriguez-Fraticelli, A.E., Ewald, A.J., Werb, Z., Alonso, M.A., and Mostov, K. (2008). Cell-polarity dynamics controls the mechanism of lumen formation in epithelial morphogenesis. *Curr Biol* 18, 507-513.
- McCaffrey, L.M., and Macara, I.G. (2011). Epithelial organization, cell polarity, and tumorigenesis. *Trends Cell Biol* 21, 727-735.
- Michel, D., Arsanto, J.P., Massey-Harroche, D., Beclin, C., Wijnholds, J., and Le Bivic, A. (2005). PATJ connects and stabilizes apical and lateral components of tight junctions in human intestinal cells. *J Cell Sci* 118, 4049-4057.
- Neufeld, S., Planas-Paz, L., and Lammert, E. (2014). Blood and lymphatic vascular tube formation in mouse. *Seminars in cell & developmental biology* 31, 115-123.
- Park, J.Y., Hughes, L.J., Moon, U.Y., Park, R., Kim, S.B., Tran, K., Lee, J.S., Cho, S.H., and Kim, S. (2016). The apical complex protein Pals1 is required to maintain cerebellar progenitor cells in a proliferative state. *Development* 143, 133-146.

- Rodriguez-Boulan, E., and Macara, I.G. (2014). Organization and execution of the epithelial polarity programme. *Nature reviews. Molecular cell biology* 15, 225-242.
- Roh, M.H., Fan, S., Liu, C.J., and Margolis, B. (2003). The Crumbs3-Pals1 complex participates in the establishment of polarity in mammalian epithelial cells. *J Cell Sci* 116, 2895-2906.
- Roh, M.H., Makarova, O., Liu, C.J., Shin, K., Lee, S., Laurinec, S., Goyal, M., Wiggins, R., and Margolis, B. (2002). The Maguk protein, Pals1, functions as an adapter, linking mammalian homologues of Crumbs and Discs Lost. *J Cell Biol* 157, 161-172.
- Ruffer, C., Strey, A., Janning, A., Kim, K.S., and Gerke, V. (2004). Cell-cell junctions of dermal microvascular endothelial cells contain tight and adherens junction proteins in spatial proximity. *Biochemistry* 43, 5360-5369.
- Schafer, R., Abraham, D., Paulus, P., Blumer, R., Grimm, M., Wojta, J., and Aharinejad, S. (2003). Impaired VE-cadherin/beta-catenin expression mediates endothelial cell degeneration in dilated cardiomyopathy. *Circulation* 108, 1585-1591.
- Schulze, C., and Firth, J.A. (1993). Immunohistochemical localization of adherens junction components in blood-brain barrier microvessels of the rat. *J Cell Sci* 104 (Pt 3), 773-782.
- Simionescu, M., Simionescu, N., and Palade, G.E. (1975). Segmental differentiations of cell junctions in the vascular endothelium. The microvasculature. *J Cell Biol* 67, 863-885.
- Simionescu, M., Simionescu, N., and Palade, G.E. (1976). Segmental differentiations of cell junctions in the vascular endothelium. Arteries and veins. *J Cell Biol* 68, 705-723.
- Straight, S.W., Shin, K., Fogg, V.C., Fan, S., Liu, C.J., Roh, M., and Margolis, B. (2004). Loss of PALS1 expression leads to tight junction and polarity defects. *Mol Biol Cell* 15, 1981-1990.
- Strilic, B., Eglinger, J., Krieg, M., Zeeb, M., Axnick, J., Babal, P., Muller, D.J., and Lammert, E. (2010). Electrostatic cell-surface repulsion initiates lumen formation in developing blood vessels. *Current biology : CB* 20, 2003-2009.
- Strilic, B., Kucera, T., Eglinger, J., Hughes, M.R., McNagny, K.M., Tsukita, S., Dejana, E., Ferrara, N., and Lammert, E. (2009). The molecular basis of vascular lumen formation in the developing mouse aorta. *Developmental cell* 17, 505-515.
- Suzuki, S., Sano, K., and Tanihara, H. (1991). Diversity of the cadherin family: evidence for eight new cadherins in nervous tissue. *Cell Regul* 2, 261-270.
- Tanihara, H., Sano, K., Heimark, R.L., St John, T., and Suzuki, S. (1994). Cloning of five human cadherins clarifies characteristic features of cadherin extracellular domain and provides further evidence for two structurally different types of cadherin. *Cell adhesion and communication* 2, 15-26.
- Tyler, R.C., Peterson, F.C., and Volkman, B.F. (2010). Distal interactions within the par3-VE-cadherin complex. *Biochemistry* 49, 951-957.

Vincent, P.A., Xiao, K., Buckley, K.M., and Kowalczyk, A.P. (2004). VE-cadherin: adhesion at arm's length. *American journal of physiology. Cell physiology* 286, C987-997.

Vorbrodt, A.W., and Dobrogowska, D.H. (2003). Molecular anatomy of intercellular junctions in brain endothelial and epithelial barriers: electron microscopist's view. *Brain Res Brain Res Rev* 42, 221-242.

Wang, Q., Hurd, T.W., and Margolis, B. (2004). Tight Junction Protein Par6 Interacts with an Evolutionarily Conserved Region in the Amino Terminus of PALS1/Stardust. *J Biol Chem* 279, 30715-30721.

Wang, Y., Kaiser, M.S., Larson, J.D., Nasevicius, A., Clark, K.J., Wadman, S.A., Roberg-Perez, S.E., Ekker, S.C., Hackett, P.B., McGrail, M., and Essner, J.J. (2010). Moesin1 and Ve-cadherin are required in endothelial cells during in vivo tubulogenesis. *Development* 137, 3119-3128.

Wu, H., Feng, W., Chen, J., Chan, L.N., Huang, S., and Zhang, M. (2007). PDZ domains of Par-3 as potential phosphoinositide signaling integrators. *Molecular cell* 28, 886-898.

Yamamoto, H., Ehling, M., Kato, K., Kanai, K., van Lessen, M., Frye, M., Zeuschner, D., Nakayama, M., Vestweber, D., and Adams, R.H. (2015). Integrin beta1 controls VE-cadherin localization and blood vessel stability. *Nature communications* 6, 6429.

Yeaman, C., Grindstaff, K.K., and Nelson, W.J. (1999). New perspectives on mechanisms involved in generating epithelial cell polarity. *Physiol Rev* 79, 73-98.

Zeeb, M., Strlic, B., and Lammert, E. (2010). Resolving cell-cell junctions: lumen formation in blood vessels. *Current opinion in cell biology* 22, 626-632.

Zegers, M.M., O'Brien, L.E., Yu, W., Datta, A., and Mostov, K.E. (2003). Epithelial polarity and tubulogenesis in vitro. *Trends Cell Biol* 13, 169-176.

Zovein, A.C., Luque, A., Turlo, K.A., Hofmann, J.J., Yee, K.M., Becker, M.S., Fassler, R., Mellman, I., Lane, T.F., and Iruela-Arispe, M.L. (2010). Beta1 integrin establishes endothelial cell polarity and arteriolar lumen formation via a Par3-dependent mechanism. *Developmental cell* 18, 39-51.

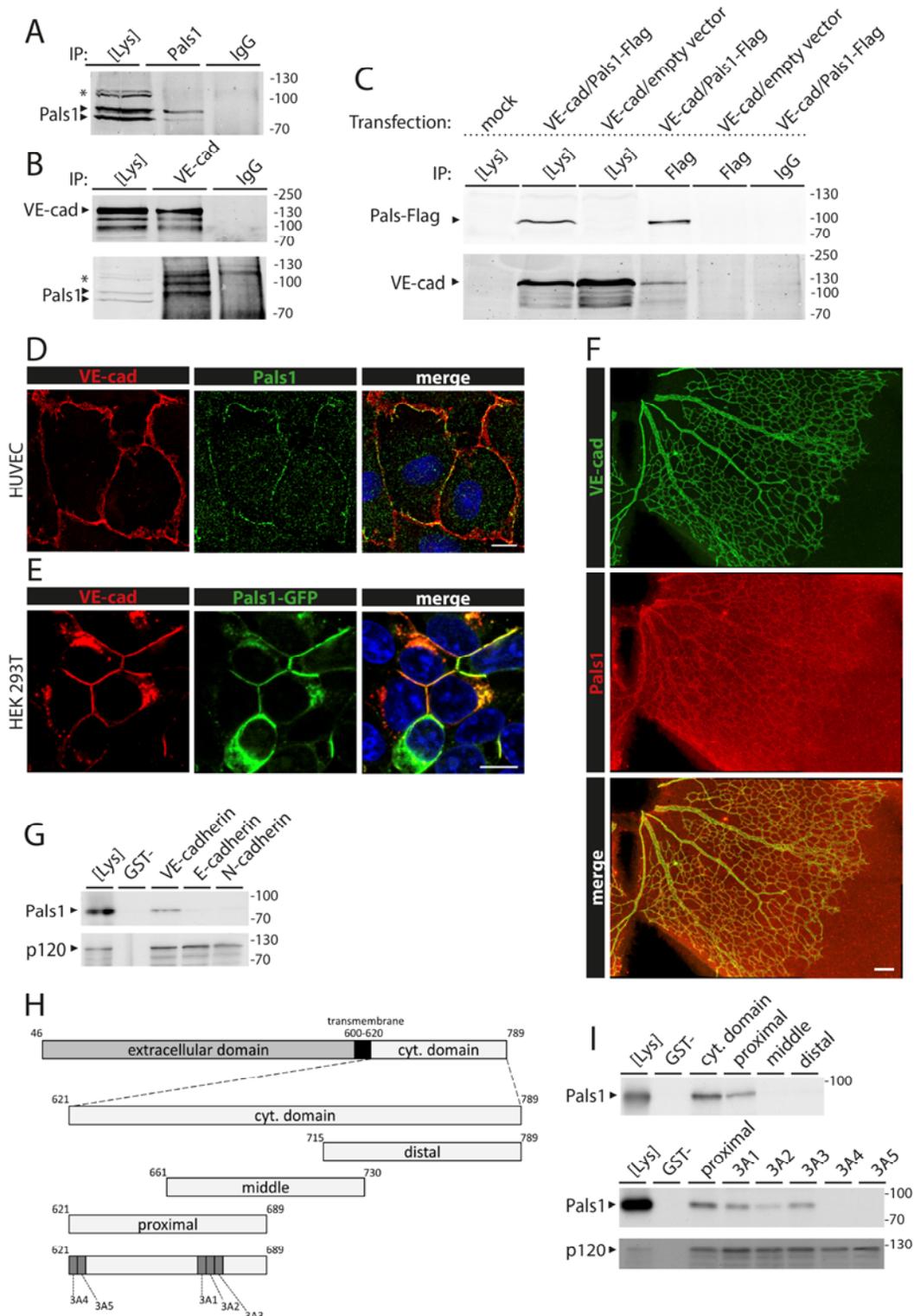


Figure 1: VE-cadherin exists in a complex with Pals1. (A) Pals1 expression in cultured endothelial cells. HUVEC lysates were analyzed for Pals1 expression by either direct western blot analysis (lane 1) or IP followed by western blot analysis (lane 2). IPs with isotype-matched control antibodies (lane 3) served as control. Pals1 appears as a doublet of

bands reflecting the 82 kDa and 72 kDa isoforms described in epithelial cells. Asterisks indicate unspecific bands. (B) Co-immunoprecipitation of Pals1 and VE-cadherin. Top panel: 10% of immunoprecipitates obtained with antibodies against VE-cadherin (VE-cad, lane 2) or with isotype-matched control antibodies (IgG, lane 3) were incubated with anti-VE-cadherin antibodies to demonstrate efficiency of VE-cadherin immunoprecipitation. Bottom panel: Immunoprecipitates obtained with antibodies against VE-cadherin were incubated with anti-Pals1 antibodies. VE-cadherin immunoprecipitates contain the 82 kDa isoform of Pals1. Lysates of HUVECs (Lys, lane 1) were loaded for comparison. Asterisks indicate unspecific bands. (C) Co-immunoprecipitation of Pals1 and VE-cadherin after ectopic expression in HEK293T cells. HEK293T cells transfected with plasmids encoding Flag-tagged Pals1 (Pals1-Flag) and VE-cadherin (VE-cad) were incubated with antibodies against Pals1. Immunoprecipitates obtained with anti-Flag antibodies were immunoblotted with antibodies against Pals1 to demonstrate efficiency of Flag-Pals1 immunoprecipitation (10% of immunoprecipitate, top panel), or with antibodies against VE-cadherin (90% of immunoprecipitate, bottom panel). Note that immunoprecipitates obtained from VE-cadherin - Pals1 double-transfected cells but not from VE-cadherin single-transfected cells contain VE-cadherin. (D) Co-localization of Pals1 with VE-cadherin at endothelial cell-cell junctions. HUVECs were stained for Pals1 and VE-cadherin as indicated. Pals1 partially co-localizes with HUVECs at cell-cell contacts (merge picture). Scale bar, 10 μm . (E) Co-localization of Pals1 and VE-cadherin after ectopic expression. HEK293T cells were transiently transfected with plasmids encoding Pals1-GFP and VE-cadherin as indicated. Pals1 and VE-cadherin co-localize at cell-cell contacts. Scale bar, 10 μm . (F) Pals1 is expressed in vascular endothelial cells in vivo. Whole-mount preparations of mouse retinas were stained with antibodies against VE-cadherin (green) and against Pals1 (red). Pals1 is expressed by vascular endothelial cells in the vascular plexus. Scale bar: 110 μm . (G) Direct interaction of Pals1 with VE-cadherin. GST fusion proteins containing the cytoplasmic domains of VE-cadherin, E-cadherin and N-cadherin were incubated with in vitro translated, ^{35}S -methionine-labeled Pals1. Pals1 interacts specifically with VE-cadherin. (H) Schematic

representation of VE-cadherin deletion and mutant constructs used in this study. (I) Pals1 interacts with a membrane-proximal motif in the cytoplasmic domain of VE-cadherin. Top panel: GST fusion proteins containing the entire cytoplasmic domain (cyt. domain) or the membrane-proximal, -middle or -distal parts of the cytoplasmic domain were incubated with in vitro translated Pals1. Pals1 interacts with the membrane-proximal domain of the VE-cadherin cytoplasmic tail. Bottom panel: GST fusion proteins containing the membrane-proximal part of the cytoplasmic domain with triple alanine (3A) mutations were analyzed for their association with in vitro translated Pals1 and p120ctn. Mutating either AA R₆₂₁-R₆₂₂-R₆₂₃ to A₆₂₁-A₆₂₂-A₆₂₃ (3A4) or AA I₆₂₄-R₆₂₅-K₆₂₆ to A₆₂₄-A₆₂₅-A₆₂₆ (3A5) (mouse VE-cadherin) abolished the association with Pals1.

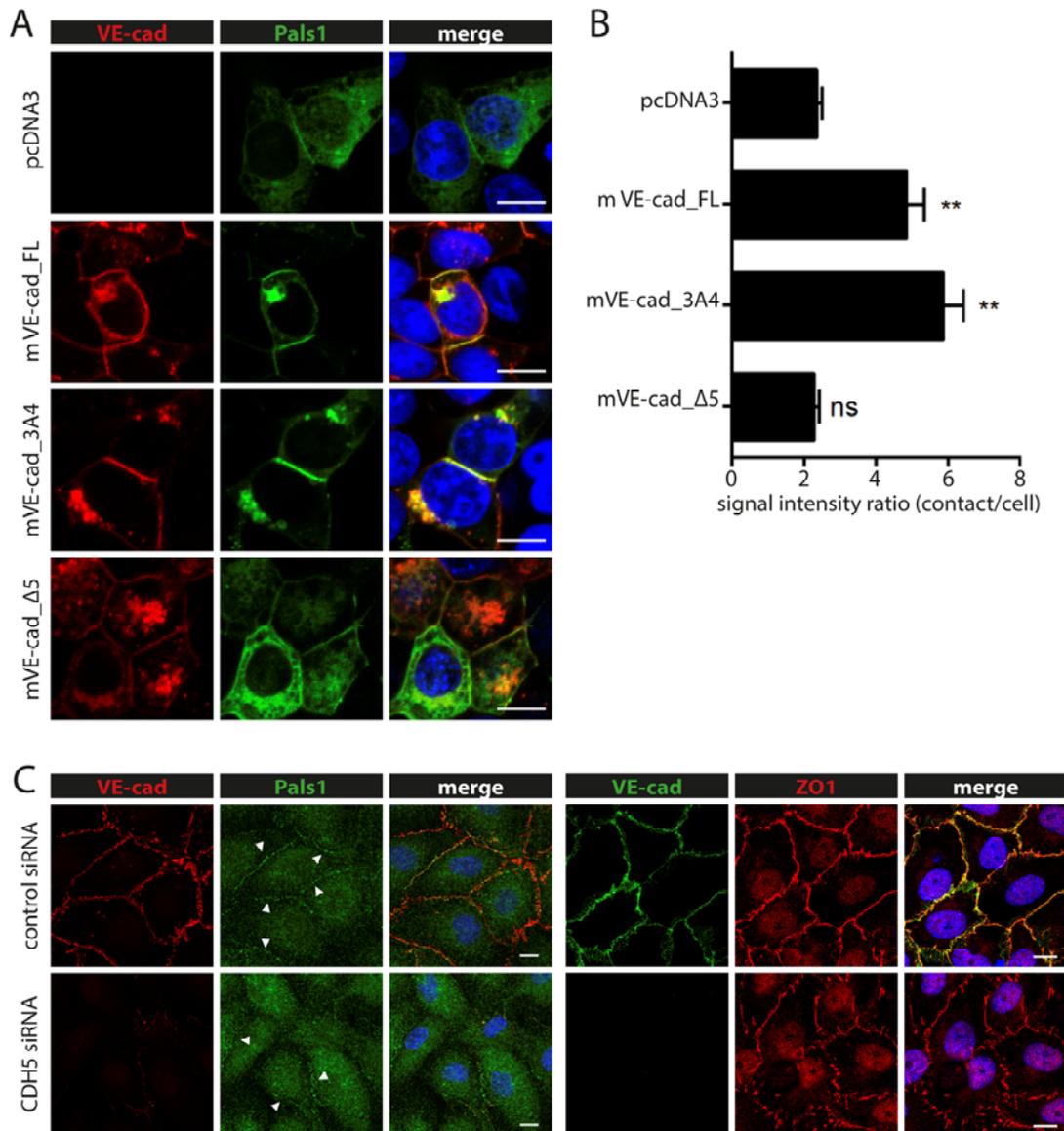


Figure 2: VE-cadherin recruits Pals1 to cell-cell contact sites. (A) Pals1 recruitment by VE-cadherin to cell-cell contacts. HEK293T cells were co-transfected with GFP-tagged Pals1 and various murine VE-cadherin constructs including full length VE-cadherin (mVE-cad_FL), the Pals1 binding mutant of VE-cadherin (mVE-cad_3A4), or the Par3 binding mutant of VE-cadherin (mVE-cad_Δ5). VE-cadherin was visualized by indirect immunofluorescence, Pals1 was visualized using GFP fluorescence. Note that VE-cadherin recruits Pals1 to cell-cell contacts, and that this activity depends on the PDZ domain-binding motif. Scale bars: 10 μm. (B) Statistical evaluation of Pals1 recruitment by VE-cadherin full length and mutant constructs. The Pals1 signal intensity is given as ratio of signal intensity at cell-cell contacts

and total signal intensities in the two contacting cells (see Method section for details). Quantitation of data shown in this figure was performed using ANOVA with Dunnett's test with 15 pictures analyzed for each condition and is presented as means \pm s.e.m.; ns, not significant, $**p < 0.0001$. (C) Pals1 localization in VE-cadherin knockdown cells. HUVECs transfected with control siRNA or VE-cadherin siRNA (CDH5 siRNA) were stained for VE-cadherin and Pals1 (left panels) or VE-cadherin and ZO-1 (right panels). Note that Pals1 localization is reduced but not completely abolished at cell-cell contacts of VE-cadherin-depleted cells. Arrowheads indicate Pals1 signals at cell-cell contact sites. Scale bars: 10 μ m.

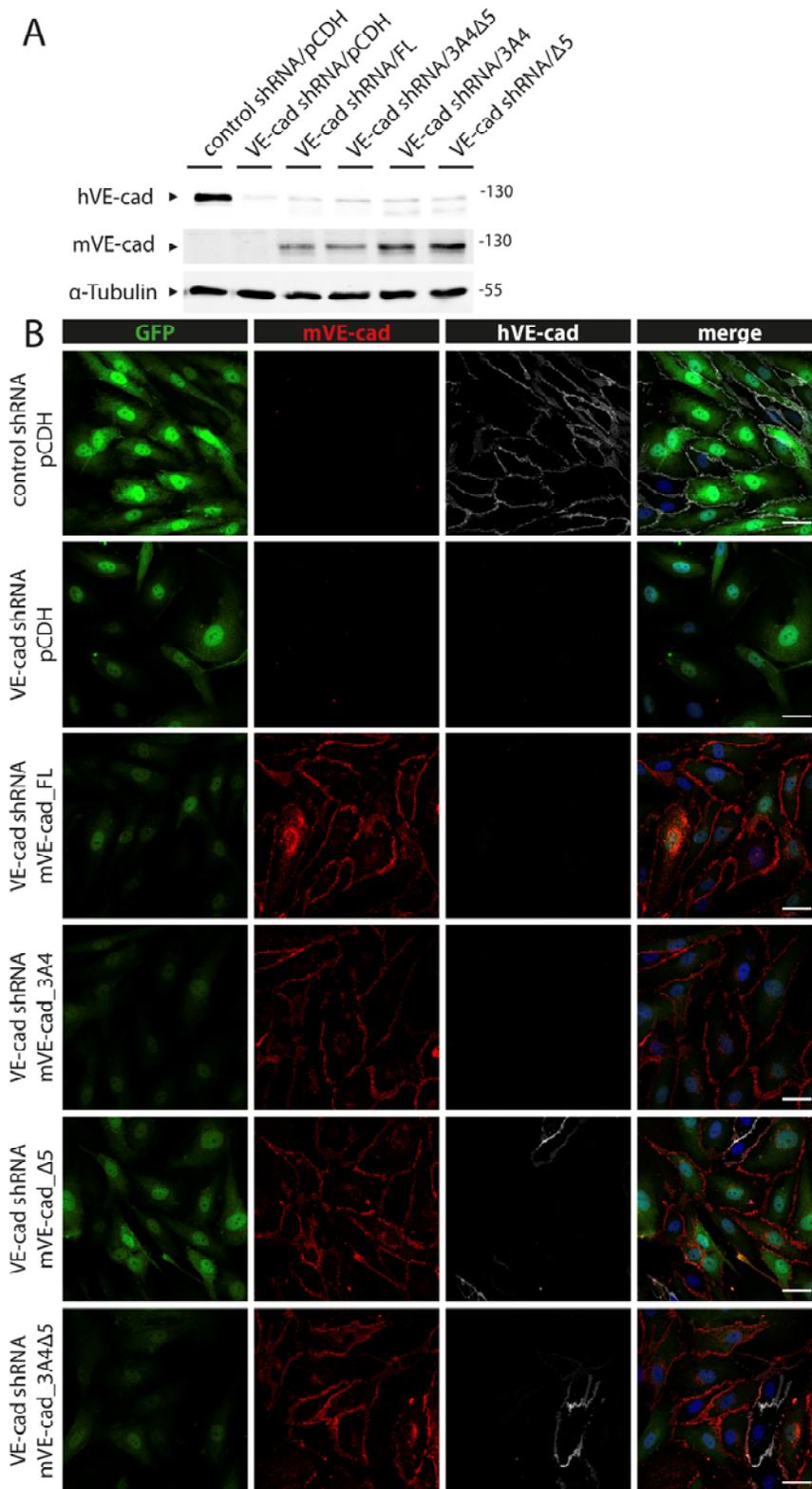


Figure 3: Replacement of endogenous VE-cadherin by VE-cadherin mutants lacking Pals1- and Par3-binding. (A) Western blot analysis of endogenous VE-cadherin and ectopically expressed mVE-cadherin (mVE-cad) constructs in HUVECs. HUVECs were

transduced with virus particles expressing either control shRNAs (control shRNA) or VE-cadherin-specific shRNAs (VE-cad shRNA) together with virus particles containing empty plasmid vectors (pCDH) or plasmid vectors encoding shRNA-insensitive mouse VE-cadherin constructs including full length VE-cadherin (FL), the Pals1/Par3 binding mutant of VE-cadherin (3A4 Δ 5), the Pals1 binding mutant of VE-cadherin (3A4), or the Par3 binding mutant of VE-cadherin (Δ 5). Lysates derived from the different cell populations were analyzed with antibodies specific for human VE-cadherin (top panel) and mouse VE-cadherin (middle panel). α -tubulin served as loading control (bottom panel). (B) Localization of endogenous VE-cadherin and ectopically expressed mouse VE-cadherin (mVE-cad) constructs in HUVECs. HUVECs transduced with lentivirus particles as described in (A) were analyzed by immunofluorescence. Transduced cells were visualized by the GFP fluorescence signal, ectopically expressed proteins were detected with antibodies specific for murine VE-cadherin (mVE-cad) or human VE-cadherin (hVE-cad). Note that all mVE-cadherin constructs localize to cell-cell contacts. Scale bar: 20 μ m.

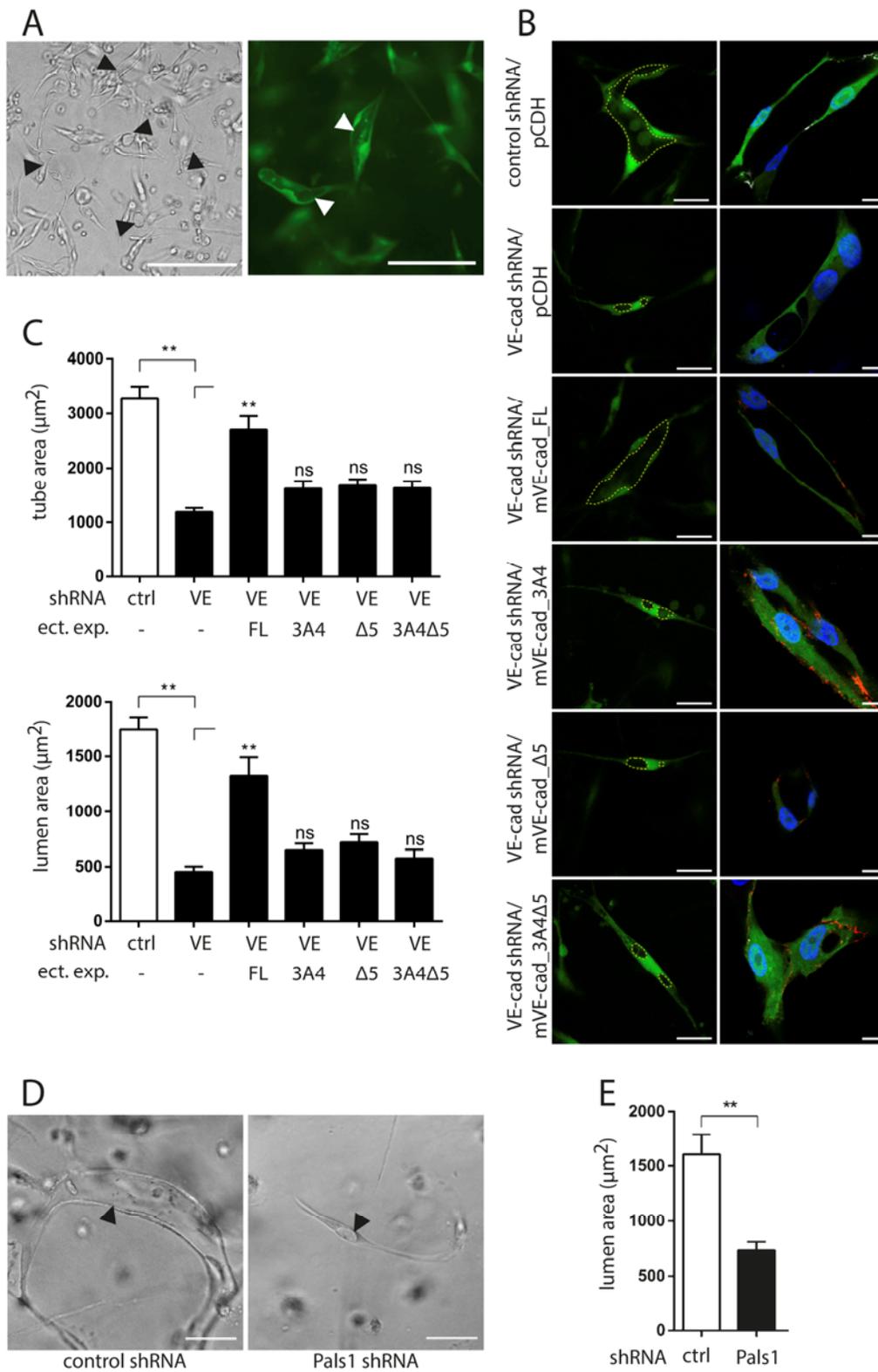


Figure 4: Spheroid formation of endothelial cells expressing Pals1- and Par3-binding mutants of VE-cadherin. (A) Spheroid formation of HUVECs grown in collagen. HUVECs

transduced with GFP-encoding lentiviral control plasmids were grown for 48 h in three-dimensional collagen gels and analyzed by phase contrast microscopy (left panel) or epifluorescence microscopy (right panel). Arrowheads point to lumen of spheroids. Scale bars: 200 μm . (B) Representative epifluorescence and confocal micrographs of spheroids developed from HUVECs transduced with VE-cadherin shRNA and mVE-cadherin cDNA constructs as indicated. For confocal imaging, spheroids were analyzed for shRNA plasmid transduction (either control shRNA or hVE-cadherin shRNA, GFP fluorescence), localization of endogenous VE-cadherin (white signal), localization of ectopically expressed mouse VE-cadherin constructs (red signal), and DNA (blue signal). Scale bars: Epifluorescence images: 50 μm , confocal images: 10 μm . (C) Statistical evaluation of spheroid formation of HUVECs transduced with VE-cadherin shRNA and mVE-cadherin cDNA constructs as indicated. Top panel: Statistical evaluation of spheroid size as analyzed by the area of the spheroid. Bottom panel: Statistical evaluation of lumen/vacuole size as analyzed by the area of the lumens/vacuoles. Quantitation of data shown in this figure was performed by one-way ANOVA followed by Dunnett's test. Data is derived from four independent experiments with 15 spheroids analyzed for each condition in each experiment. Data is presented as means \pm s.e.m.. ns, not significant, **p < 0.0001. (D) Spheroid formation of HUVECs after Pals1 depletion. Representative bright field microscopy images of spheroids developed from control shRNA transduced HUVECs (left panel) and Pals1 shRNA transduced HUVECs (right panel) transfected. Scale bars: 50 μm . (E) Statistical evaluation of lumen/vacuole size after Pals1 depletion as analyzed by the area of the lumens/vacuoles. Quantitation of data shown in this figure was performed by one-way ANOVA followed by Dunnett's test. Data is derived from three independent experiments with a minimum of 15 spheroids analyzed for each condition in each experiment. Data is presented as means \pm s.e.m. ns, not significant, **p < 0.0001.

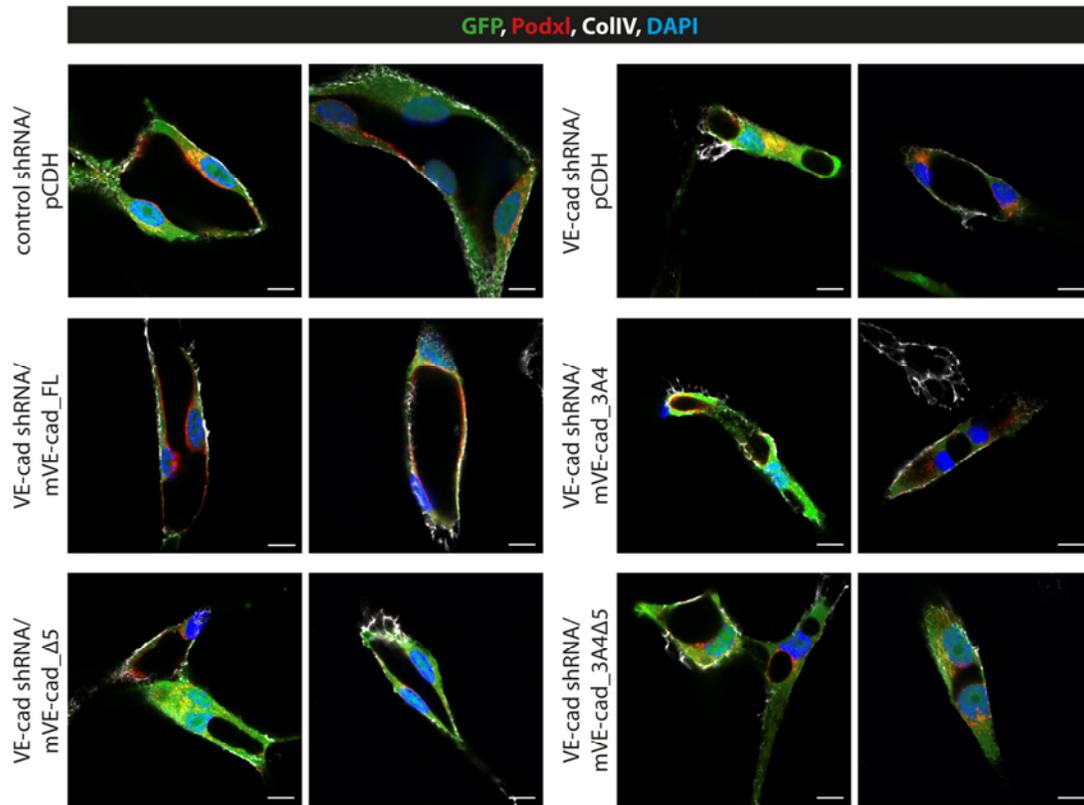


Figure 5: Membrane polarity of 3D-grown endothelial cells expressing Pals1- and Par3-binding mutants of VE-cadherin. HUVECs with endogenous VE-cadherin replaced by Pals1 or Par3 or Pals1/Par3 binding mutants were grown for 48 h in 3D-collagen gels. Spheroids were stained with antibodies against podocalyxin (Podxl) and collagen type IV (ColIV) to stain the apical and basal membrane domains, respectively. Lentivirally transduced cells are visualized by GFP fluorescence, nuclei are visualized with DAPI. Representative confocal images of HUVEC spheroids are shown for each construct. Scale bars: 10 μm.

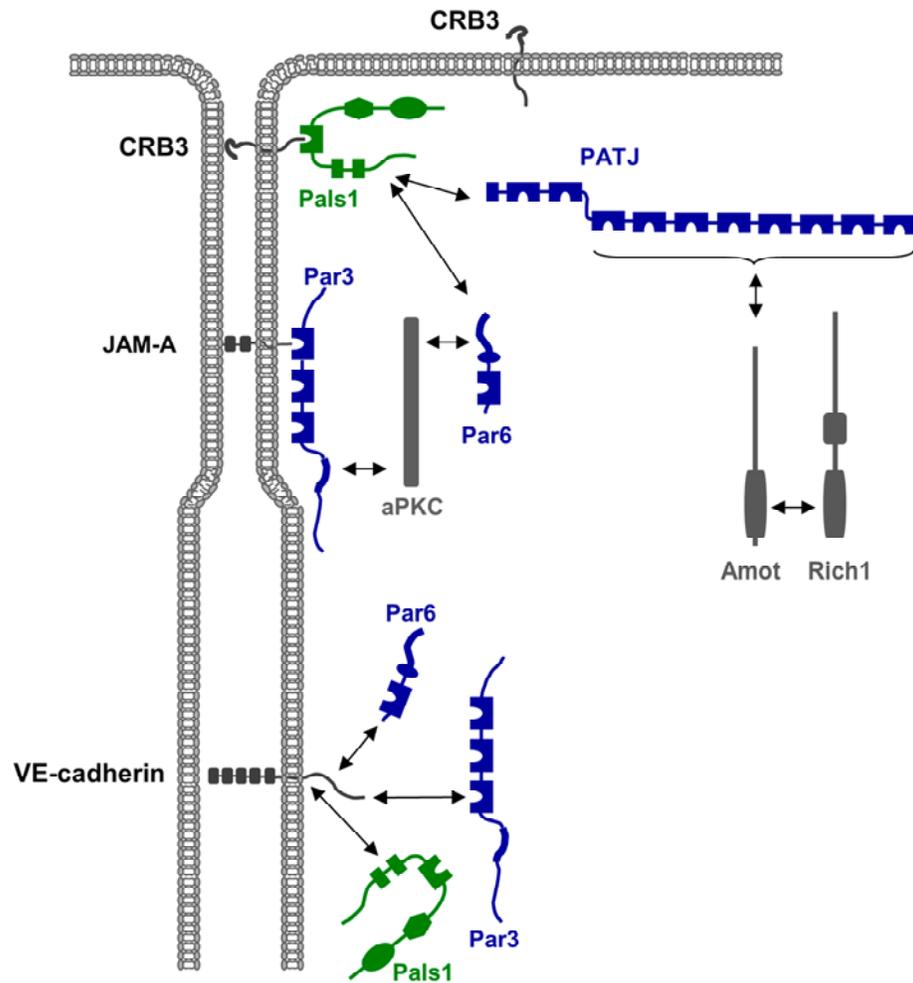


Figure 6: The Pals1 interaction network. Pals1 directly interacts with several scaffolding proteins localized at tight junctions and adherens junctions including PATJ and Par6. In addition, Pals1 directly interacts with transmembrane proteins CRB3 and VE-cadherin. Direct interactions are indicated by double arrows. Note that the interactions of Pals1 with CRB3, Par6 and PATJ have been demonstrated in epithelial cells, the interaction with VE-cadherin is specific for endothelial cells.