Planar Cell Polarity Protein Celsr1 Regulates Endothelial Adherens Junctions and Directed Cell Rearrangements during Valve Morphogenesis

Florence Tatin, Andrea Taddei, Anne Weston, Elaine Fuchs, Danelle Devenport, Fadel Tissir, and Taija Makinen

Inventory of Supplemental Data

Supplemental Figures

Supplemental Figure S1
Related to Figure 1. Cell shape analysis of Prox1\textsuperscript{high} valve-forming cells in the developing lymphatic vessels.

Supplemental Figure S2
Related to Figure 2. Expression of the PCP core proteins Celsr1, Vangl2 and Frizzled 6 in the developing and mature lymphatic valves.

Supplemental Figure S3
Related to Figure 4. Characterization of valve morphology in mesenteric lymphatic vessels in mice lacking Celsr1, Celsr2 or Celsr3.

Supplemental Figure S4
Related to Figure 5. Characterization of the cell-cell junction organization and maturation in primary lymphatic endothelial cells.

Supplemental Figure S5
Related to Figure 5. Still images from two time-lapse movies of primary LECs transfected with Celsr1-GFP showing dynamic behavior of Celsr1-expressing membrane filopodia and cells.

**Supplemental Figure S6**
Related to Figure 5. Localization of Celsr1 and junctional components in primary LECs.

**Supplemental Figure S7**
Related to Figure 6. Analysis of VE-cadherin internalization in Celsr1 positive and negative lymphatic endothelial cells.

**Supplemental Movies**

**Supplemental Movie S1**
Related to Figure 1. 3D reconstruction of a developing valve in E17.5 mesenteric lymphatic vessel from images of serial semi-thin sections.

**Supplemental Movie S2**
Related to Figure 5. Time-lapse movie of primary LECs transfected with Celsr1-GFP showing dynamic behaviour of Celsr1-positive filopodia on membrane protrusions between two Celsr1-expressing cells.

**Supplemental Movie S3**
Related to Figure 5. Time-lapse movie of primary LECs transfected with membrane bound YFP showing dynamic behaviour of filopodia on membrane protrusions between LECs.

**Supplemental Movie S4**
Related to Figure 5. Time-lapse movie of Celsr1-GFP transfected LECs showing recruitment of Celsr1 to cell-cell contacts in between Celsr1 expressing cells.
Supplemental Figure S1
Supplemental Figure S3

| mature (V-shape) | immature (ring-shape) | no rotation | random orientation |
|------------------|-----------------------|-------------|--------------------|
| A  | B  | C  | D  |

A'  | A''  | Celsr1  | Prox1  | Celsr1 |
| B'  | B''  | Celsr1  | Prox1  | Celsr1 |

E  | F  | G  |

E18.5  | E18.5  | E18.5 |

H  | H'  | I  |

E17.5  | E17.5  | E17.5 |
Supplemental Figure S5
Supplemental Figure S6
Supplemental Figure legends

**Figure S1, related to Figure 1. Cell shape analysis of Prox1\textsuperscript{high} valve-forming cells in the developing lymphatic vessels.**

(A-C) Visualization of the cell shape by mosaic labeling of lymphatic endothelial cells (marked by mGFP fluorescence (green) in Prox1-CreER\textsuperscript{T2};R26-mTmG vessels) and of the nuclear shape by Prox1 staining (purple) at E16.5. (A) shows tile scan image in which areas magnified in (B, C) are indicated. Single channel images for GFP (A’-C’) and Prox1 (A’’-C’’) are shown. Note the elongated shape of Prox1\textsuperscript{high} valve-forming cells (arrows).

Scale bars: 100 µm.

**Figure S2, related to Figure 2. Expression of the PCP core proteins in lymphatic valves.**

(A) Whole-mount immunofluorescence of mesenteric lymphatic vessels for Celsr1 (green), Vangl2 (red) and PECAM-1 (blue) at the indicated stages. Single channel images for Celsr1 and Vangl2 are shown. Dotted lines outline lymphatic vessels.

(B) Whole-mount immunofluorescence of mesenteric lymphatic vessels for Frizzled 6 (green) and PECAM-1 (red).

(C) Visualization of Celsr1 (purple) in individually labeled lymphatic endothelial cells (marked by mGFP fluorescence (green) in Prox1-CreER\textsuperscript{T2};R26-mTmG vessels) at E16.5. Single channel image for Celsr1 is shown on the right. Note the localization of Celsr1 to the tips of filopodia (arrowheads).

(D) Orientation of Celsr1-positive filopodia in E16.5 mesenteric vessels (n=99 filopodia from 9 vessels). Flow direction is indicated.

Scale bars: 40 µm (A, B), 30 µm (C)
Figure S3, related to Figure 4. Characterization of valve morphology in Celsr1, Celsr2 and Celsr3 mutants.

(A-D) Whole-mount immunofluorescence of E18.5 control (A) and Celsr1^{-/-} (B-D) mesenteric vessels for Celsr1 (green), Prox1 (red) and PECAM-1 (blue). Single channel images for Celsr1 (A’-D’) and Prox1 (A’’-D’’) are shown. Three different categories were described: mature valves (V-shape; A), immature valves (ring-shape; B) and abnormal valves showing no rotation (C) or misalignment/random orientation (D) of Prox1^{high} cells. Note an abnormal cluster of Prox1^{high} cells in a valve that was assigned to the category ‘ring-shape’ in the Celsr1 mutant (arrow in B).

(E-G) Whole-mount immunofluorescence of E18.5 Celsr2^{-/-} (E), Celsr3^{-/-} (F) and Celsr1^{-/-};Celsr3^{-/-} (G) mesenteric vessels for Prox1.

(H-I) Measurement of Prox1^{high} cell orientation in E17.5 control (H) and Celsr1^{-/-} (I) mesenteric vessels. The angle between the longitudinal axis of the vessel (while line) and the axis of the cell (red line) was measured (H’). 0° = longitudinal, 90° = perpendicular alignment to the axis of the vessel. Dotted lines outline lymphatic vessels.

Scale bars: 40 µm (A-I), 80 µm (H’)

Figure S4, related to Figure 5. Cell-cell junction organization and maturation in lymphatic endothelial cells.

(A, B) Quantification of the morphology (A) and the width of membrane overlap at cell-cell contacts (B) over time in primary LECs. Data in (A) represent the percentage of overlapping contacts (> 3.10 µm) and linear junctions (< 3.10 µm). Data in (B) represent the absolute and average (red bar) width of n=150 junctions per time point.
(C) Immunostaining for VE-cadherin (green), PECAM-1 (red) and DAPI (blue). Note the gradual enrichment of VE-cadherin during the maturation of cell-cell junctions (arrows). Single channel images for VE-cadherin are shown below.

(D) Visualization of cell-cell junction organization in confluent LECs and BECs by VE-cadherin (green) and F-actin (red) staining. Single channel images are shown on the right. Note cortical actin associated with VE-cadherin in the LECs while the BECs display stress fibers and overlapping membrane contacts.

Scale bars: 20 µm (C), 40 µm (D)

Figure S5, related to Figure 5. Time-lapse imaging of Celsr1-GFP expressing lymphatic endothelial cells.

(A) Still images from a time-lapse movie of primary LECs transfected with Celsr1-GFP showing dynamic behaviour of Celsr1-positive filopodia (arrowheads) at the indicated time-points.

(B) Still images from a time-lapse movie that was started 4h after plating of Celsr1-GFP transfected cells at the indicated time-points. Arrows show the recruitment of Celsr1 to cell-cell contacts in between two cells sliding over each other. Arrowhead shows Celsr1-positive contact between cells moving away from each other. Asterisks indicate cells expressing Celsr1-GFP. Dotted lines outline the cells.

Scale bar: 10 µm (A), 20 µm (B)

Figure S6, related to Figure 5. Celsr1 does not co-localize with components of the adherens or tight junctions in primary lymphatic endothelial cells.

(A-F) Localization of Celsr1-GFP (green) and indicated junctional components (red) in primary LECs.
Scale bars: 40 μm (A-F), 20 μm (A’-D’’, F’-F’’), 5 μm (E’-E’’).

**Figure S7, related to Figure 6. Celsr1 does not regulate VE-cadherin internalization.**
Visualization of VE-cadherin (red staining) internalization in LECs transfected with Celsr1-GFP (green) in presence or absence of chloroquine. Quantification of VE-cadherin positive vesicles in chloroquine-treated cells (arrow) is shown on the right. Data represent the average number of vesicles per cell ±SEM (n=27 control cells; n=20 Celsr1-GFP positive cells).
Scale bars: 40 μm

**Movie S1, related to Figure 1. 3D reconstruction of a developing valve in E17.5 mesenteric lymphatic vessel.** Blue colour highlights valve endothelial cells forming a disc inside the vessel lumen and grey represents the vessel wall. Reconstruction was done using images of serial semi-thin sections (400 nm) through the developing valve.

**Movie S2, related to Figure 5. Dynamic behaviour of Celsr1-positive filopodia on membrane protrusions.** Time-lapse movie of primary LECs transfected with Celsr1-GFP showing dynamic behaviour of Celsr1-positive filopodia on membrane protrusions between two Celsr1-expressing cells. Confocal images were acquired every 1 min and six z-stack images at 0.349 μm intervals were merged.

**Movie S3, related to Figure 5. Visualization of membrane protrusions with membrane-bound-YFP at cellular junctions.** Time-lapse movie of primary LECs transfected with CAAX-YFP showing dynamic behaviour of filopodia on membrane protrusions. Confocal images were acquired every 1 min and seven z-stack images at 0.349 μm intervals were merged.
Movie S4, related to Figure 5. Recruitment of Celsr1 to cell-cell contacts in between Celsr1 expressing cells. Time-lapse movie was started 4h after plating of Celsr1-GFP transfected cells. Confocal images were obtained every 10 min for overnight and seven z-stack images at 1 µm intervals were merged.
Supplemental experimental procedures

Mouse strains

The following mouse strains that have been described previously were used: Prox1-CreER\textsuperscript{T2} (Bazigou et al., 2011), R26-mTmG (Muzumdar et al., 2007), Looptail ((Kibar et al., 2001), provided by Dr. D. Savery and Dr. A Copp, University College London), Celsr2\textsuperscript{+/−} (Tissir et al., 2010) and conditional Celsr1\textsuperscript{flax} (Ravni et al., 2009) and Celsr3\textsuperscript{flax} (Tissir et al., 2005). In the text Celsr1\textsuperscript{flax/flax};Prox1-CreER\textsuperscript{T2} and Celsr1\textsuperscript{flax/flax};Celsr3\textsuperscript{flax/flax};Prox1-CreER\textsuperscript{T2} lines are denoted as Celsr1\textsuperscript{+/−} and Celsr1\textsuperscript{+/−};Celsr3\textsuperscript{+/−}, respectively. Characterization of cell shapes in Figure 1 was done using a Prox1-CreER\textsuperscript{T2} founder line that gave mosaic labelling of lymphatic endothelia. In all other experiments a founder line that was described in (Bazigou et al., 2011) was used.

Immunofluorescence

For whole-mount immunofluorescence, tissues were fixed for one hour in 4% PFA. Before staining, the samples were permeabilized in PBST (PBS, 0.3% TritonX-100) and blocked in PBST plus 3% milk. Primary antibodies were used at the following dilutions: guinea pig anti-mouse Celsr1 ((Devenport and Fuchs, 2008), 1/200), rabbit anti-mouse Vangl2 ((Montcouquiol et al., 2006), provided by Dr. M. Montcouquiol, Neurocentre Magendie - Université Bordeaux, 1/250), rat anti-mouse PECAM-1 (BD Biosciences, 1/250), hamster anti-mouse PECAM-1 (Chemicon, 1/200), rabbit anti-mouse Claudin-5 (Zymed, 1/100), goat anti-VE-cadherin (Santa Cruz, 1/100) rabbit anti-human Prox1 (Abcam, 1/100, or generated against human Prox1 C-terminus (567-737aa), Prox1-GST construct provided by Dr. T. Petrova, University of Lausanne), goat anti-mouse Foxc2 and Integrin-α9 (both from R&D Systems, 1/100), rabbit anti-mouse Laminin-α5 ((Ringelmann et al., 1999), provided by Dr.
L. Sorokin, University of Muenster, 1/400) and mouse anti-human Fibronectin EIIIA (FN-3E2, Sigma, 1/400). Secondary antibodies (Jackson ImmunoResearch, 1/350) were incubated for 2h at room temperature in PBST. After washing, the samples were mounted in Mowiol. For immunofluorescence of cells, the cells grown on glass coverslips were fixed in 4% PFA, washed in PBS and permeabilized in 0.5% TritonX-100. Primary antibodies were used at the following dilutions in blocking solution (2% FBS, 3% BSA in PBS): goat anti-human VE-cadherin (Santa Cruz, 1/200), rabbit anti-human VE-Cadherin (Bender Medsystems, 1/200), pMLC (Cell Signalling, 1/200), MIIB (Cell Signalling, 1/100), ZO-1 and Claudin-5 (Invitrogen, 1/100), PAR6 (Santa Cruz, 1/25), PAR3 (Millipore, 1/50) or mouse anti-human PECAM-1 (DakoCytomation, 1/100). F-actin was visualized by Rhodamine conjugated Phalloidin.

Quantitative analysis

For quantification of the number of valves, mesenteries from control (n=7), Celsr1+/− (n=10) and Celsr1−/−;Celsr3−/− (n=5) were stained for Prox1 and PECAM-1, and three categories of valve morphology were defined: mature valves (V-shape), immature valves (ring-shape) and abnormal valves (no rotation of Prox1high cells). For quantification of the shape and the orientation of Prox1high nuclei, E17.5 control (Cre-negative littermate; n=9) and Celsr1−/− (n=10) valves from 3 independent experiments (150 Prox1high cells per genotype in total) were analyzed. Cell shape was calculated by measuring nucleus length/width ratio using Image J software. Cell orientation was calculated by measuring the rotation angles of Prox1high cells with respect to the longitudinal axis of the vessel using angle tools in Image J software. Tile scan images with Plan Apochromat DIC 63×/1.4 NA oil objective were used for quantification of filopodia orientation. The orientation of filopodia was determined using
filaments tools in Imaris Bitplane software and Matlab software was used to generate Rose Plot.

For the analysis of junctional maturation in cultured primary LECs, membrane protrusions and linear junctions were measured at their broadest point using ImageJ software. Junctions broader than 3.10 µm were classified as overlapping contacts, those thinner than 3.10 µm as linear junctions.

**Time-lapse imaging**

Transfected LECs were seeded on glass bottom culture dishes (MatTek Corporation) that were pre-coated with Fibronectin. To visualise Celsr1-GFP at cell junctions (Movie S2) and membrane-bound-YFP (Movie S3), confocal time-lapse images were acquired every 1 min using Plan-Apochromat 63x/1.46 NA Oil Korr and z-stack images at 0.349 µm intervals were merged. For the visualization of dynamic behaviour of cells 4h after plating (Movie S4), confocal images were obtained every 10 min for overnight using C-Apochromat 40x/1.1 NA W Korr and seven z-stack images at 1 µm intervals were merged.

**Assays for VE-cadherin internalization and cell-cell junction formation**

24 hours after transfection, LEC were incubated at 37°C in the presence of 300 µM chloroquine (Sigma) for 4.5 hours to allow visualization of endogenous VE-cadherin internalization, followed by fixing and immunofluorescence as described. For the analysis of junctional organization of LECs at different states of confluence, 5000 cells/cm² were plated and cultured for 7 days. Cells were fixed in 4% PFA at every 24h and processed for immunofluorescence as described. De novo junction formation was assessed by plating transfected LECs in ECGMV2 medium containing 0.5% of supplement and 3mM EGTA, in
order to inhibit calcium-dependent cell-cell contact formation prior to the expression of Celsr1-GFP. 5 hours later EGTA was removed and cells were cultured for another 5 hours in EGTA-free medium to allow junction formation. Cells were fixed in 4% PFA and processed for immunofluorescence as described.
Supplemental references

Bazigou, E., Lyons, O.T., Smith, A., Venn, G.E., Cope, C., Brown, N.A., and Makinen, T. (2011). Genes regulating lymphangiogenesis control venous valve formation and maintenance in mice. J Clin Invest 121, 2984-2992.

Kibar, Z., Vogan, K.J., Groulx, N., Justice, M.J., Underhill, D.A., and Gros, P. (2001). Ltap, a mammalian homolog of Drosophila Strabismus/Van Gogh, is altered in the mouse neural tube mutant Loop-tail. Nat Genet 28, 251-255.

Muzumdar, M.D., Tasic, B., Miyamichi, K., Li, L., and Luo, L. (2007). A global double-fluorescent Cre reporter mouse. Genesis 45, 593-605.

Ravni, A., Qu, Y., Goffinet, A.M., and Tissir, F. (2009). Planar cell polarity cadherin Celsr1 regulates skin hair patterning in the mouse. J Invest Dermatol 129, 2507-2509.

Tissir, F., Bar, I., Jossin, Y., De Backer, O., and Goffinet, A.M. (2005). Protocadherin Celsr3 is crucial in axonal tract development. Nat Neurosci 8, 451-457.

Tissir, F., Qu, Y., Montcouquiol, M., Zhou, L., Komatsu, K., Shi, D., Fujimori, T., Labeau, J., Tyteca, D., Courtoy, P., et al. (2010). Lack of cadherins Celsr2 and Celsr3 impairs ependymal ciliogenesis, leading to fatal hydrocephalus. Nat Neurosci 13, 700-707.