Claudin-like protein 24 interacts with the VEGFR-2 and VEGFR-3 pathways and regulates lymphatic vessel development

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The Claudin-like protein of 24 kDa (CLP24) is a hypoxia-regulated transmembrane protein of unknown function. We show here that *clp24* knockdown in *Danio rerio* and *Xenopus laevis* results in defective lymphatic development. Targeted disruption of *Clp24* in mice led to enlarged lymphatic vessels having an abnormal smooth muscle cell coating. We also show that the *Clp24*$^{-/-}$ phenotype was further aggravated in the *Vegfr2*$^{+/LacZ}$ or *Vegfr3*$^{+/LacZ}$ backgrounds and that CLP24 interacts with vascular endothelial growth factor receptor-2 (VEGFR-2) and VEGFR-3 and attenuates the transcription factor CREB phosphorylation via these receptors. Our results indicate that CLP24 is a novel regulator of VEGFR-2 and VEGFR-3 signaling pathways and of normal lymphatic vessel structure.

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The lymphatic vasculature is required for tissue fluid balance and immune system function (Tammela and Alitalo 2010). Dysfunction of the lymphatic system can lead to lymphedema, characterized by swelling of extremities due to fluid accumulation in tissues. Lymphatic vessels also represent the primary route of metastatic spread for many types of human cancers, and they are intimately involved in various inflammatory disorders (Tammela and Alitalo 2010).

The development of the lymphatic vasculature in mice starts at around embryonic day 10.5 (E10.5), after the formation of a functional cardiovascular system (Adams and Alitalo 2007; Tammela and Alitalo 2010). Vascular endothelial growth factor-C (VEGF-C) induces lymphatic sprouting by activating the VEGF receptor-3 (VEGFR-3) on the surface of the first differentiated lymphatic endothelial cells (Karkkainen et al. 2004). VEGFR-3 is expressed initially in all endothelial cells of mouse embryos, but becomes restricted to the lymphatic endothelium during development (Tammela and Alitalo 2010). Some lymphangiogenic signals are also mediated by VEGFR-2, which is expressed in blood vessels, collecting lymphatic vessels, and also lymphatic capillaries undergoing lymphangiogenesis; for example, in tumors (Nagy et al. 2002; Hirakawa et al. 2005; Wirzenius et al. 2007). The further remodeling and maturation of the lymphatic vasculature requires several other molecules (e.g., Nrp2, podoplanin, Foxc2, Syk, SLP76, Angpt2, and Angptl4) (Adams and Alitalo 2007; Tammela and Alitalo 2010).

In order to better understand the development of the lymphatic vasculature, we searched for genes involved in lymphangiogenesis by carrying out a genome-wide gene expression analysis of primary human lymphatic endothelial cells (LECs) and blood vascular endothelial cells (BECs). Here we report on one of the genes enriched in LECs, the hypoxia-inducible Claudin-like protein of 24 kDa (*CLP24*; *TMEM204*). We show that CLP24 is essential for lymphatic development in zebrafish, frogs, and mice. Interestingly, CLP24 interacted with VEGFR-2 and VEGFR-3 and attenuated VEGF- and VEGF-C-induced phosphorylation of the transcription factor CREB, suggesting that CLP24 is a modulator of VEGFR-2 and VEGFR-3 signals required for lymphatic vascular development.

Results and Discussion

Endothelial expression of CLP24

To search for genes enriched in LECs, we compared the gene expression profiles of cultured LECs and BECs from human skin microvasculature using oligonucleotide arrays. We identified the *CLP24* mRNA, which was expressed more abundantly in LECs than BECs cultured in vitro (Supplemental Fig. S1A) and from freshly ex vivo isolated microvascular endothelium (LEC vs. BEC fold: 5.8×, P = 0.004) (Wick et al. 2007). All tested human tissues except the bone marrow and peripheral blood contained *CLP24* mRNA of 1.9 kb, with enhanced levels in highly vascularized tissues such as the heart, lung, kidney, adrenal gland, and placenta (Supplemental Fig. S1B). We confirmed that *CLP24* is a hypoxia-regulated gene (Supplemental Fig. S1C; Kearsey et al. 2004). *CLP24* was conserved in all species, including humans, mice, zebrafish, and frogs (Supplemental Fig. S1D,E).

We found that most of the *Clp24* expression occurred in blood vessels at E10.5, E15.5, and E16.5 (Fig. 1A–D; Supplemental Figs. S2A–D, S3). In a screen of novel

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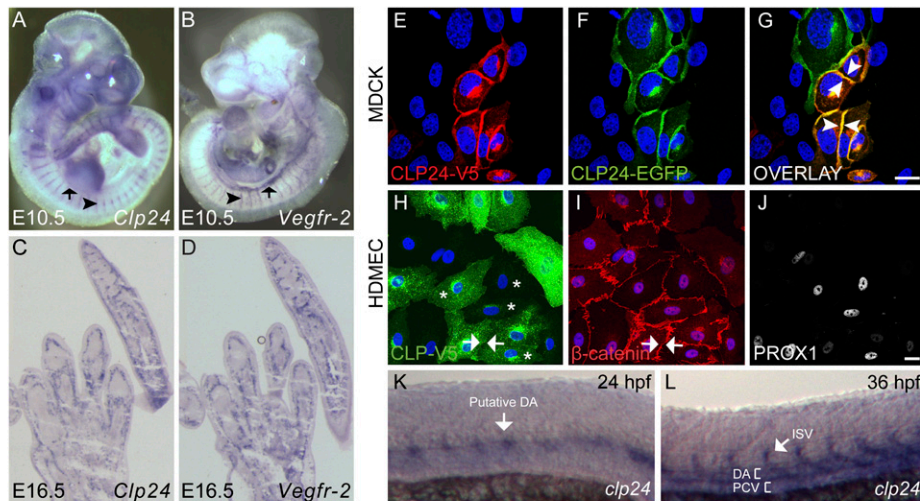

Figure 1. Endothelial expression of CLP24. (*A*,*B*) Whole-mount ISH of E10.5 mouse embryos. *Clp24* is expressed in ISVs similarly to *Vegfr2*. Arrows indicate the cardinal vein, and arrowheads indicate the ISVs. (*C*,*D*) *Clp24* ISH of E16.5 mouse hindleg and tail. (*E*–*G*) MDCK cells transfected with CLP24-EGFP and CLP24-V5 retroviruses were stained with anti-V5 antibody. (*H*–*J*) HDMECs transfected with CLP24-EGFP (*H*) retrovirus were stained with anti-β-catenin (*I*) and anti-PROX1 (*J*) antibodies to identify LECs (asterisks in *H*). Nuclei are stained with DAPI. CLP24 is localized to cell–cell junctions in epithelial cells (arrowheads in *G*), but not in endothelial (arrows in *H*,*I*) cells. (*K*,*L*) *clp24* ISH of *D. rerio* embryos shows expression in the vasculature at the indicated hours post-fertilization (hpf). (DA) Dorsal aorta. Bar, 20 μm.

cardiac genes, the *Clp24* transcript was detected previously in the developing vascular system before E9.5 (Christoforou et al. 2008). Notably, the expression pattern of *Vegfr2* was very similar to that of *Clp24*. At E10.5, *Clp24* and *Vegfr2* were both observed in, e.g., intersomitic vessels (Fig. 1A,B, arrowheads), while only *Vegfr2* was detected in larger vessels, such as the cardinal vein (Fig. 1A,B, arrows). At E10.5, *Clp24* was also detected in the developing limb buds and branchial arches. At E15.5 and E16.5, *Clp24* and *Vegfr2* were prominent in the blood vessels, e.g., in the brain and developing limb bud (Fig. 1C,D; Supplemental Figs. S2A–D, S3). However, *Clp24* mRNA was absent from the neural retina, where *Vegfr2* was expressed (Supplemental Fig. S3, arrowheads).

CLP24 has been suggested to be a distant member of the claudin family of transmembrane proteins, which are engaged in homotypic interactions across the cell–cell junctions (Kearsey et al. 2004). We confirmed that overexpressed CLP24 is localized at cell–cell junctions in transfected Madin-Darby canine kidney (MDCK) epithelial cells, but not human dermal microvascular endothelial cells (HDMECs), where CPL24 was distributed uniformly at the plasma membrane in LECs and BECs (Fig. 1E–J; Supplemental Fig. S2E,F).

clp24 *is required for lymphatic vessel development in* Danio rerio *and* Xenopus laevis

In order to elucidate the biological function of CLP24, we studied it in zebrafish and frog embryos. In situ hybridization (ISH) on early embryos showed that the *D. rerio clp24* homolog (*tmem204*) was expressed initially in the dorsal aorta, and later also in the posterior cardinal vein (PCV) and intersegmental vessels (ISVs) (Fig. 1K,L). The knockdown of *clp24* using 6–8 ng of morpholino (MO) directed against the 5′ untranslated region (UTR) of *clp24* mRNA caused a subtle blood vascular defect, characterized by abnormal extra branching of the ISVs, but only from 4 d post-fertilization (dpf) onward, thus after the initiation of lymphatic development (Fig. 2A–D). The most striking defect was the impaired formation of the lymphatic thoracic duct (TD) (6 dpf) and its immediate precursor structure, the parachordal lymphangioblasts (48 h post-fertilization [hpf]) in *clp24* morphants. The penetrance and severity of these defects were dose-dependent (Fig. 2E,F), and the results were confirmed using a second *clp24* MO targeted against the translation start site (Supplemental Fig. S4A,B). Thus, the striking lymphatic defect occurred prior to the appearance of the subtle blood vascular defects.

Twenty nanograms to 60 ng of the *clp24* MO resulted in lymphatic and blood vascular defects of *X. laevis* embryos in a dose-dependent manner (Supplemental Fig. S4A). Live screening at stage 45 (Fig. 2G,H) showed edema in the heart, gut, and cloaca region in 58% of the *clp24* morphants versus 7% of control MO-injected tadpoles ($P < 0.0001$), and blood flow arrest in 32% of *clp24* morphants versus 3% of controls ($P < 0.0001$) despite normal beating of the heart and lymph hearts. In addition, 21% of the *clp24* morphants had blood spots in their tissues (versus 0% of controls; $P < 0.0001$).

To further characterize the phenotypes, knockdown of *clp24* was performed in transgenic *Tg(Flk1:eGFP)* frogs that express eGFP in their blood and lymphatic vessels. Neither the ventral caudal lymphatic vessel (VCLV) nor the dorsal caudal lymphatic vessel (DCLV) assembled into a compact lymph vessel, and the LECs appeared dispersed and disorganized (Fig. 2I,J) in the *clp24* morphants. Lymphangiography showed that only 12.5% of the morphants ($n = 8$) were able to take up and drain injected dye via the VCLV, as compared with 100% ($n = 11$) of the control embryos (Fig. 2K,L).

Staining for the lymphatic marker *prox1* (Ny et al. 2005) at stage 35/36 revealed decreased commitment (−19%) toward the lymphatic lineage at the level of the PCV (prox1$^+$ area: 35,800 ± 1266 μm^2 in control tadpoles [$n = 69$] vs. 29,100 ± 1392 μm^2 in *clp24* morphants [$n = 58$, $P = 0.001$]) (Fig. 2M,N). Fewer prox1-positive cells were

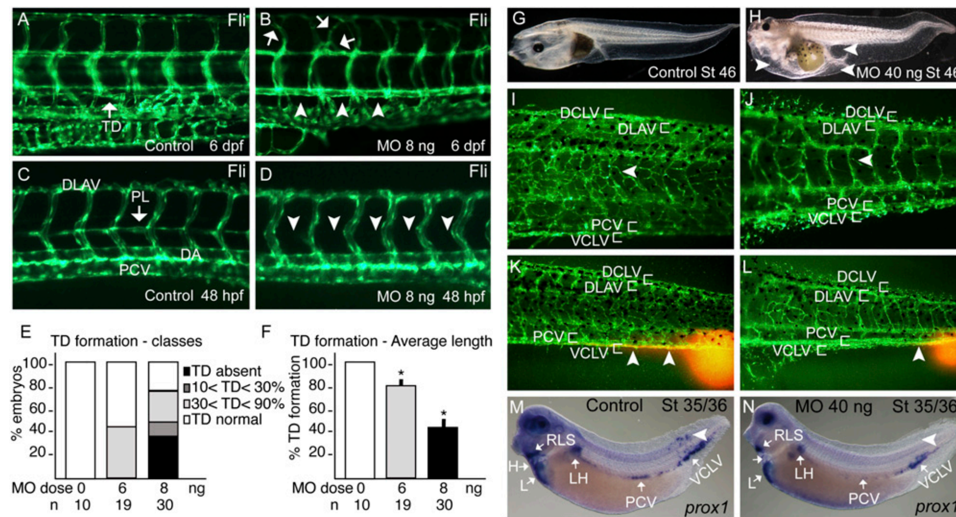

Figure 2. Clp24 is required for vascular and lymphatic development in *D. rerio* and *X. laevis*. (*A*–*D*) Control-injected (*A*,*C*) and *clp24* MO-injected (*B*,*D*) embryos of a fluorescent zebrafish (*Fli1:eGFP^{y1}*) line were screened at 6 dpf (*A*,*B*) and 48 hpf (*C*,*D*). Note extra branching of the ISVs (arrows in *B*) and absence of TD (arrowheads in *B*) at 6 dpf, and absence of parachordal lymphatic precursors (PL) (arrow in *C*) in *clp24* morphants (arrowheads in *D*). (*E*,*F*) TD formation in *clp24* morphants. (*E*) Percentage of affected embryos. (*F*) Average TD length over 10-somite tail segment at 6 dpf. (*) $P < 0.05$ versus control. (*G*,*H*) At stage 46, c*lp24* MO-treated (*H*) but not control-treated (*G*) *X. laevis* tadpoles had massive edema in heart, gut, and cloaca region (arrowheads). (*I*–*L*) Transgenic *Tg(Flk1:eGFP) X. laevis* embryos were injected with 40 ng of *clp24* (*J*,*L*) or control MO (*I*,*K*). (*I*,*J*) Small capillaries, but not the main blood vessels, were to a large extent missing in *clp24* morphants (arrowheads). (*J*) Both the VCLV and the DCLV failed to assemble into a compact lymph vessel, and the LECs appear dispersed and disorganized. (*K*,*L*) Lymphangiography for MO-treated tadpoles. Note the absence of dye uptake by the malformed VCLV in *clp24* morphants (arrowhead, *L*) as compared with control (arrowheads, *K*). (*M*,*N*) Control (*M*) and *clp24* (*N*) MO-treated *X. laevis* embryos were analyzed at stage 35/36 by *prox1* ISH. The *clp24* morphants show less staining in the PCV, heart (H), and rostral lymph sac (RLS), and in the area of the future lymph hearts (LH) and of the future VCLV. Note a reduced number of prox1-positive cells migrating across the tail from the VCLV to form the DCLV (arrowheads). Staining in liver (L) seems similar to control. (DA) Dorsal aorta; (DLAV) dorsal longitudinal anastomosing vessel.

migrating dorsally across the tail (–25%) to form the DCLV in *clp24* morphants (prox1$^+$ area of migration: $20{,}520 \pm 1113\ \mu m^2$ in control tadpoles vs. $15{,}370 \pm 1223\ \mu m^2$ in *clp24* morphants; $P = 0.003$). Furthermore, ISH for the blood vessel marker *msr* showed reduced numbers of ISV sprouts in the *clp24* morphants (Supplemental Fig. S4C–F).

Clp24 *gene targeted mice have enlarged lymphatic vessels with abnormal smooth muscle cell (SMC) coverage*

In order to reveal the in vivo function of *Clp24* in mammals, we produced gene targeted *Clp24* mice in which the first exon of the gene was flanked by loxP sites (Supplemental Fig. S5). We crossed the *Clp24*$^{lox/lox}$ mice with the PGK-Cre mice to delete *Clp24* in all tissues (Lallemand et al. 1998). *Clp24*$^{-/-}$ mice were born in a normal Mendelian ratio, and survived until adulthood with no obvious health problems. However, immunohistochemical analysis of the vasculature of the *Clp24*$^{-/-}$ mice showed enlarged lymphatic vessels in various organs (Fig. 3A–E; Supplemental Figs. S6, S8I, S9C,D), while the blood capillary network appeared similar to that in wild-type littermates (Supplemental Fig. S9A,B,E–H).

Closer analysis of the ear vasculature showed that the collecting lymphatic vessels located in the deeper layers of the ears of the *Clp24*$^{-/-}$ mice were dilated when compared with wild-type littermates, but were equally covered by a continuous layer of SMC actin (SMA)-positive perivascular support cells (Fig. 3D,E; Supplemental Fig. S6C,D, arrowheads). In the more superficial layers of the ear, the lymphatic vessels gradually lose the SMC coating, and express LYVE-1 (Supplemental Fig. S6B,D). However, in the *Clp24*$^{-/-}$ mice, the enlarged initial lymphatic vessels had increased numbers of associated SMCs when compared with wild type (Fig. 3F; Supplemental Fig S6A–D, arrows). Notably, LYVE-1 expression was down-regulated in the *Clp24*$^{-/-}$ lymphatic vessels in the sites that were in contact with SMCs, suggesting that SMCs modulated the lymphatic vessel identity (Supplemental Fig S6A, arrow).

We also mated the *Clp24*$^{lox/lox}$ mice with the Tie1-Cre mice, which results in deletion of *Clp24* in endothelial cells. Importantly, the lymphatic phenotype of the endothelial cell-specific *Clp24*$^{-/-}$ knockout mice (*Clp24EC*$^{-/-}$) was similar to that of the *Clp24*$^{-/-}$ mice (Supplemental Fig. S7), strongly suggesting that endothelial cell-expressed *Clp24* specifically contributes to the formation of the lymphatic vasculature.

CLP24 interacts with the VEGFR-2 and VEGFR-3 pathways in lymphatic vessel patterning

In order to analyze possible involvement of CLP24 in VEGFR-2 or VEGFR-3 pathways, we mated the *Clp24*$^{-/-}$ mice with *Vegfr2*$^{+/LacZ}$ and *Vegfr3*$^{+/LacZ}$ mice. The enlarged lymphatic vessels of the *Clp24*$^{-/-}$ mice were even more dilated in the *Vegfr3*$^{+/LacZ}$ and *Vegfr2*$^{+/LacZ}$ backgrounds (Supplemental Fig. S8A–F). Notably, normal-sized lymphatic vessels of the *Clp24*$^{+/-}$ mice were dilated upon deletion of one allele of either *Vegfr2* or *Vegfr3*. Staining for β-galactosidase showed no abnormalities in the blood vasculature of the *Clp24*$^{-/-}$;*Vegfr2*$^{+/LacZ}$ mice (Supplemental

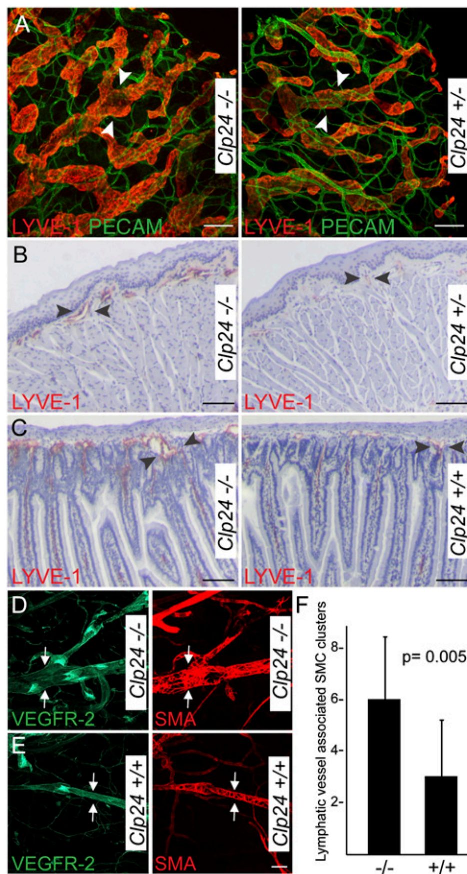

Figure 3. Abnormal lymphatic vessels in the *Clp24* gene targeted mice. (*A*) Whole-mount staining of lymphatic and blood vessels for LYVE-1 and PECAM-1, respectively, in the ears of $Clp24^{-/-}$ and $Clp24^{+/-}$ mice. (*B*,*C*) Paraffin sections of the tongue (*B*) and the small intestine (*C*) of wild-type, $Clp^{+/-}$, and $Clp24^{-/-}$ mice were stained for LYVE-1. Note enlarged lymphatic vessels in $Clp24^{-/-}$ mice (arrowheads in *A*–*C*). (*D*,*E*) Whole-mount staining of the ears of $Clp24^{-/-}$ (*D*) and wild-type ($Clp24^{+/+}$) (*E*) mice for VEGFR-2 and SMA to detect perivascular SMCs. Note enlarged SMC-covered collecting lymphatic vessels (arrows) in $Clp24^{-/-}$ mice. (*F*) Quantification of SMC-covered initial lymphatic vessels in the ear periphery in $Clp24^{-/-}$ and wild-type mice. Shown is the mean of lymphatic vessel-associated SMC clusters per 2.25-mm^2 field. Representative images used for quantification are shown in Supplemental Figure S6, C and D. Bar, 100 μm.

Fig. 9E–H), but decorated abnormal lymphatic vessels of the $Clp24^{-/-}$;$Vegfr3^{+/LacZ}$ mice (Supplemental Fig. S8G–I). Similarly, down-regulation of *clp24* in *Xenopus* embryos in combination with *vegfr3* down-regulation led to an aggravation of the lymphatic phenotype (Supplemental Fig. S10). These results indicated that *Clp24* interacts with the *Vegfr2* and *Vegfr3* pathways in lymphatic vessel patterning.

We next analyzed any possible interactions between CLP24 and VEGFR-2 or VEGFR-3. CLP24 was found to coimmunoprecipitate with both VEGFR-2 and VEGFR-3 from double-transfected human umbilical vein endothelial cells (HUVECs) and 293T cells, in both the presence and absence of ligand stimulation (Fig. 4A,B; data not shown). As a negative control, CLP24 did not coimmunoprecipitate with Tie-2 from double-transfected cells (Fig. 4C).

To further explore the cellular mechanism behind the lymphatic phenotype of the $Clp24^{-/-}$ mice, we analyzed lung microvascular endothelial cells isolated from the $Clp24^{-/-}$ mice. $Clp24^{-/-}$ endothelial cells showed normal morphology and expression of endothelial cell-specific proteins (Supplemental Fig. S11). Ligand stimulation of VEGFRs leads to activation of several downstream signaling molecules—in particular, strong phosphorylation of the transcription factor CREB (Heckman et al. 2008). The VEGF-induced phosphorylation of CREB was increased significantly in $Clp24^{-/-}$ endothelial cells when compared with wild-type cells, while other signaling proteins, such as p38MAPK, were unaltered (Fig. 4D–I; data not shown). The VEGFR-2- and VEGFR-3-specific ligands VEGF-E and VEGF-C156S, respectively, induced CREB phosphorylation in the LECs (Fig. 4J–O), which was reduced significantly in CLP24-transfected cells (Fig. 4N,O). These experiments indicated that CLP24 modulates signaling via both VEGFR-2 and VEGFR-3.

Our results show that CLP24 is essential for lymphatic development in frogs, zebrafish, and mice. The lymphatic vessels of the $Clp24^{-/-}$ mice were dilated and showed abnormal recruitment of SMCs, while in the frog and zebrafish, the lymphatic development appeared to fail altogether. In zebrafish, the parachordal lymphangioblasts were absent from the horizontal myoceptum at 48 hpf, and in stage 35/36 tadpoles, the prox1-positive cells did not bud dorsally from the PCV to form the DCLV. Thus, the early stages of lymphatic development were abrogated in both fish and frogs, while in mice the initial phases of lymphatic development occurred, and vessel abnormalities were detected later. This suggests that, in mice, CLP24 may act during the later stages of lymphatic differentiation, when the SMC-covered collecting lymph vessels are demarcated from lymph capillaries (Tammela and Alitalo 2010). More SMC-covered enlarged lymphatic vessels have also been described in mice deficient of the Foxc2 transcription factor or angiopoietin-2, or having a mutant ephrinB2 (Petrova et al. 2004; Makinen et al. 2005; Dellinger et al. 2008). Although *clp24* knockdown caused mild blood vascular defects in zebrafish and frog embryos, such were not found in the $Clp24^{-/-}$ mice.

It remains to be studied why the fish and frog vasculatures are more susceptible to the loss of *clp24* when compared with mice. Although there are no close homologs of *Clp24* in mice, it is possible that a compensating mechanism allows $Clp24^{-/-}$ mice to pass early lymphatic development, thus explaining the differences between the mouse versus the frog and fish phenotypes. In addition, collecting lymphatic vessels have not been reported in fish, and part of the phenotype we describe (difference in SMC recruitment) is associated with their differentiation. It should also be noted that VEGF-C has a more widespread role in fish compared with mice. In mice, VEGF-C is required for all lymphatic sprouting during development, and it also contributes to retinal and tumor angiogenesis (Karkkainen et al. 2004; Tammela et al. 2008), while the zebrafish VEGF-C is also required for venous-derived angiogenesis in the trunk (Hogan et al. 2009). *Clp24* knockdown in the fish and frog prevented lymphangiogenic budding from venous endothelium, a process critically dependent on VEGF-C (Hogan et al. 2009). It is possible that CLP24 participates also in VEGF-C-mediated blood vascular development in fish and frog embryos, thus explaining the observed vascular defects in the morphants.

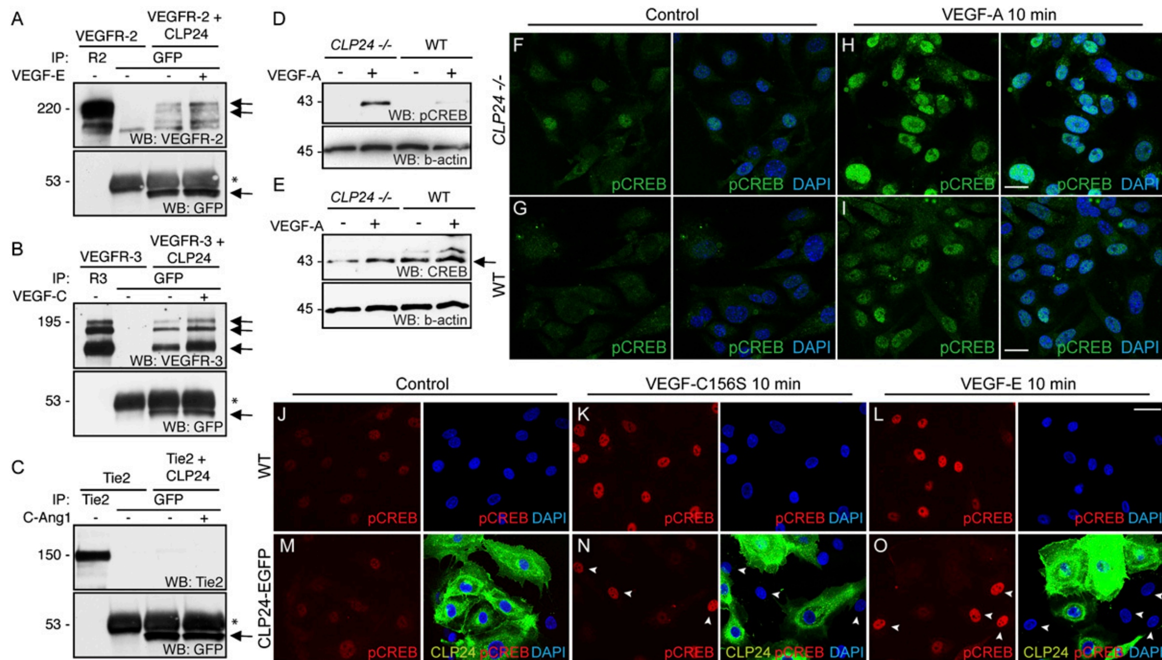

Figure 4. CLP24 interacts physically with VEGFR-2 and VEGFR-3 and modulates CREB phosphorylation. (*A*–*C*) HUVECs transfected with CLP24-EGFP together with VEGFR-2 (*A*), VEGFR-3 (*B*), or Tie2 (*C*) retrovirus were starved; stimulated 15 min with either VEGF-E (*A*), VEGF-C (*B*), or COMP-Ang1 (*C*); and used for immunoprecipitation of CLP24-EGFP followed by anti-VEGFR-2 (*A*), anti-VEGFR-3 (*B*), or anti-Tie2 (*C*) immunoblotting and reprobing using anti-GFP antibody. CLP24 coimmunoprecipitated with VEGFR-2 (*A*) and VEGFR-3 (*B*), but not with Tie2. The asterisk shows migration of Ig light chain. (*D*,*E*) Endothelial cells from wild-type or *Clp24*$^{-/-}$ mice were stimulated with VEGF, and were analyzed by immunoblotting for phospho-CREB (pCREB) (*D*), total CREB (*E*), and β-actin (for equal loading). (*F*–*I*) Wild-type (*G*,*I*) or *Clp24*$^{-/-}$ (*F*,*H*) endothelial cells were stimulated with VEGF and stained for pCREB. (*J*–*O*) Untransfected (*J*–*L*) and CLP24-EFGP retrovirus transfected (*M*–*O*) intestinal LECs were stimulated with VEGF-C156S (*K*,*N*) or VEGF-E (*L*,*O*), and were stained for pCREB. CREB is phosphorylated in endothelial cells after stimulation of VEGFR-3 (*K*) and VEGFR-2 (*L*), and at higher levels in untransfected cells (arrowheads) than in CLP24-EGFP-expressing cells (*N*,*O*). Nuclei were stained with DAPI.

The lymphatic phenotype of *Clp24*$^{-/-}$ mice was more pronounced when one allele of *Vegfr2* or *Vegfr3* was also deleted. Interestingly, CLP24 modulated signaling through VEGFR-2 and VEGFR-3, as phosphorylation of CREB was enhanced when *Clp24* was deleted, but was reduced when CLP24 was overexpressed. CREB phosphorylation has been linked to increased cell survival (Lee et al. 2009), which could partly explain the observed enlargement of lymphatic vessels in the *Clp24*$^{-/-}$ mice. We found that CLP24 coimmunoprecipitated with VEGFR-2 and VEGFR-3 from endothelial cells, suggesting a possibility that CLP24 directly regulates VEGFR-2/VEGFR-3 function. However, further studies are required to unravel the mechanism of CLP24-mediated modulation of VEGFR-2/VEGFR-3 signaling.

Overexpression of VEGFs is associated with pathological activation of the VEGFR-2 and VEGFR-3 signaling pathways. Blocking of the VEGF–VEGFR-2 pathway is the first anti-angiogenic therapy that is being increasingly used to treat human diseases (Crawford and Ferrara 2009). The present study discovers CLP24 as a novel modulator of VEGFR-2 and VEGFR-3 signaling, and indicates that CLP24 has an essential function in the lymphatic vasculature, while the possible role of CLP24 in pathological angiogenesis remains to be studied. In conclusion, our results suggest that different effectors are recruited to VEGFR-2 and VEGFR-3 to control the diverse downstream functions of VEGF/VEGF-C signaling in lymphatic versus blood vessels.

Materials and methods

Cell culture, reagents, immunoblotting, immunofluorescence

Cell culture, retrovirus production, immunoblotting, and immunofluorescence are described previously (Saharinen et al. 2008), and antibodies and growth factor stimulations are described in the Supplemental Material. TIE2, VEGFR-2, VEGFR-3, CLP24-EGFP, and CLP24-V5 cDNAs were cloned into pMXs vector (a generous gift from Dr. Toshio Kitamura, University of Tokyo). Mouse lung endothelial cells were isolated as explained previously (Reynolds and Hodivala-Dilke 2006) and in the Supplemental Material.

ISH and scoring of TD and parachordal lymphatic precursor formation in zebrafish embryos

Clp24 ISH was performed as described (Chittenden et al. 2006), and live screening and quantification of TD formation was performed as detailed in the Supplemental Material.

ISH, MO injections, and morphometric and general analysis of the development in X. laevis

Whole-mount ISH using *prox1* probe was performed as described previously (Ny et al. 2005) and in the Supplemental Material. The generation and characterization of the *Tg(Flk1:eGFP)* line will be reported elsewhere. The cloning of *clp24*, the *X. laevis clp24* ortholog, and the MO oligos are detailed in the Supplemental Material.

Mouse models

The *Vegfr3*$^{+/lz}$ (Dumont et al. 1998), *Vegfr2*$^{+/lz}$ (Shalaby et al. 1995), Tie1-Cre (Gustafsson et al. 2001), and PGK-Cre (Lallemand et al. 1998) mice

were used. The conditional *Clp24* gene targeted mice were produced at GenOway (http://www.genoway.com). Immunohistochemistry, whole-mount staining, and LacZ staining of mouse tissues were done as in Petrova et al. (2004).

ISH of mouse tissues

ISH was performed using digoxigenin-labeled probes on 16- to 30-μm frozen sections of mouse embryos as detailed in the Supplemental Material.

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