Context-dependent functions of angiopoietin 2 are determined by the endothelial phosphatase VEPTP

Tomokazu Souma[a,b,1], Benjamin R. Thomson[a,b,1], Stefan Heinen[c,1], Isabel Anna Carota[a,b], Shinji Yamaguchi[a,b], Tuncer Onay[a,b], Pan Liu[a,b], Asish K. Ghosh[a], Chengjin Li[d], Vera Eremina[d], Young-Kwon Hong[e], Aris N. Economides[f], Dietmar Vestweber[g], Kevin G. Peters[h], Jing Jin[a,b], and Susan E. Quaggin[a,b,2]

[a]Feinberg Cardiovascular Research Institute, Northwestern University Feinberg School of Medicine, Chicago, IL 60611; [b]Division of Nephrology/Hypertension, Northwestern University Feinberg School of Medicine, Chicago, IL 60611; [c]Hurvitz Brain Sciences Program, Sunnybrook Research Institute, Toronto, ON MN4 3M5, Canada; [d]Lunenfeld-Tanenbaum Research Institute, Mount Sinai Hospital, Toronto, ON M5G 1X5, Canada; [e]Keck School of Medicine, University of Southern California, Los Angeles, CA 90033; [f]Regeneron Pharmaceuticals, Tarrytown, NY 10591; [g]Max Planck Institute of Molecular Biomedicine, 48149 Münster, Germany; and [h]Aerpio Therapeutics, Cincinnati, OH 45242

Edited by Kari Alitalo, Wihuri Research Institute and University of Helsinki, Helsinki, Finland, and approved December 20, 2017 (received for review August 20, 2017)

The angiopoietin (ANGPT)–TIE2/TEK signaling pathway is essential for blood and lymphatic vascular homeostasis. ANGPT1 is a potent TIE2 activator, whereas ANGPT2 functions as a context-dependent agonist/antagonist. In disease, ANGPT2-mediated inhibition of TIE2 in blood vessels is linked to vascular leak, inflammation, and metastasis. Using conditional knockout studies in mice, we show TIE2 is predominantly activated by ANGPT1 in the cardiovascular system and by ANGPT2 in the lymphatic vasculature. Mechanisms underlying opposing actions of ANGPT2 in blood vs. lymphatic endothelium are poorly understood. Here we show the endothelial-specific phosphatase VEPTP (vascular endothelial protein tyrosine phosphatase) determines TIE2 response to ANGPT2. VEPTP is absent from lymphatic endothelium in mouse in vivo, permitting ANGPT2/TIE2-mediated lymphangiogenesis. Inhibition of VEPTP converts ANGPT2 into a potent TIE2 activator in blood endothelium. Our data support a model whereby VEPTP functions as a rheostat to modulate ANGPT2 ligand effect on TIE2.

angiopoietin–TIE2 pathway | VEPTP | angiogenesis | lymphangiogenesis | tyrosine kinase

The angiopoietin–TIE2 (tyrosine kinase with Ig and EGF homology domains, also known as TEK) receptor tyrosine kinase pathway regulates vascular homeostasis, maturation, and remodeling, and has been described as the "gatekeeper" of vascular quiescence (1–4). TIE2 phosphorylation enhances vascular stability by promoting endothelial cell survival, reducing responsiveness to inflammatory stimuli, and strengthening cellular junctions in mature vessels (5–10). The primary TIE2 agonist, angiopoietin 1 (ANGPT1), is secreted from perivascular cells, acting in a paracrine manner (7, 11). A second ligand, ANGPT2, has been described as a context-dependent agonist/antagonist, despite having similar receptor affinity as the agonistic ligand ANGPT1 (12–14). Unlike ANGPT1, ANGPT2 is secreted by endothelial cells and acts on the TIE2 receptor in an autocrine manner (15).

The ANGPT–TIE2 pathway has attracted attention due to strong associations and causal links with human diseases, including rare genetic disorders, such as hereditary vascular malformations and primary congenital glaucoma (16–18), as well as common diseases, such as sepsis, cancer, diabetes, and cardiovascular disease (7, 19, 20). Vigorous efforts have been made to understand and translate this pathway to the clinic. However, a major question remains: why does ANGPT2 display opposing context-dependent roles in different vascular beds (14)? In blood endothelial cells (BECs), ANGPT2 is described as an antagonist of ANGPT1-mediated TIE2 activation (1, 3, 12, 14), while the situation is reversed in lymphatic endothelial cells (LECs), where ANGPT2 serves as the primary TIE2 agonist (21).

In the blood endothelium, where TIE2 signaling plays an important role in vascular stability, elevated levels of circulating ANGPT2 in vascular diseases, such as sepsis, result in TIE2 inhibition, leading to increased capillary leakiness and poor clinical outcomes (2, 19). Developmental mouse models provide further support for ANGPT2-mediated antagonism of ANGPT1–TIE2 activation in blood vessels (1, 3, 4). Deletion of either *Angpt1* or *Tie2* results in embryonic lethality at embryonic day (E) 10.5 due to severe defects in cardiovascular development (7–11). This phenotype is reproduced by endothelial overexpression of *Angpt2*, supporting an antagonistic role for ANGPT2 in the blood endothelium (12).

In contrast to its antagonistic role in the blood vasculature, ANGPT2 functions as a TIE2 agonist in the lymphatic endothelium (1, 3, 21). *Angpt2* knockout mice display lymphatic defects, including chylous ascites, as well as a sprouting defect in the retinal blood vascular capillaries (22–24). Intriguingly, only the lymphatic phenotypes were rescued by the obligate TIE2 agonist ANGPT1, supporting an agonistic role for ANGPT2 specific to the lymphatic endothelium (22–24).

Two recent papers have suggested that TIE2 is not required for lymphatic function in vivo, raising questions about the mechanism of ANGPT2-mediated lymphangiogenesis (25, 26). However, here we report that LEC-specific loss of TIE2 phenocopies the lymphatic defects observed in *Angpt2* knockout mice, confirming that TIE2 is required for lymphatic development. Based on these data,

Significance

Reducing vascular leakage and stabilizing the endothelium through activation of the angiopoietin (ANGPT)–TIE2 receptor tyrosine kinase pathway is a promising therapeutic strategy for vascular diseases. ANGPT2 is one of two major ligands for the TIE2 receptor. Uniquely, ANGPT2 possesses an agonistic role in lymphatic endothelium, but acts as a competitive antagonist in blood endothelium. The molecular basis for the opposing actions of ANGPT2 in these two vascular beds is poorly understood. Here we demonstrate that the absence of VEPTP expression in the lymphatic endothelium confers an agonist function of ANGPT2 on TIE2 receptor, but VEPTP expression in blood endothelium abrogates its activity. Our findings provide mechanistic insights needed to advance therapeutic targeting of this pathway.

Author contributions: T.S., B.R.T., S.H., J.J., and S.E.Q. designed research; T.S., B.R.T., S.H., I.A.C., S.Y., T.O., P.L., A.K.G., C.L., and V.E. performed research; Y.-K.H., A.N.E., D.V., K.G.P., and J.J. contributed new reagents/analytic tools; T.S., B.R.T., S.H., J.J., and S.E.Q. analyzed data; and T.S., B.R.T., S.H., J.J., and S.E.Q. wrote the paper.

Conflict of interest statement: A.N.E. is an employee of Regeneron Pharmaceuticals. K.G.P. is an employee of Aerpio Therapeutics.

This article is a PNAS Direct Submission.

[1]T.S., B.R.T., and S.H. contributed equally to this work.

[2]To whom correspondence should be addressed. Email: quaggin@northwestern.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1714446115/-/DCSupplemental.

we hypothesize that the context-dependent agonist/antagonist function of ANGPT2 and its opposing effects on TIE2 in different vascular beds (i.e., LEC vs. BEC) might be explained by differential expression of molecular components of the pathway, including negative regulators, such as the endothelial specific phosphatase, vascular endothelial protein tyrosine phosphatase (VEPTP) (27–30).

To elucidate the molecular basis of opposing functions of ANGPT2 in LECs vs. BECs, we generated a series of gene-modified mouse lines and determined a critical cell-autonomous role for TIE2 signaling in lymphangiogenesis. We found that VEPTP is absent from LECs but abundant in BECs, and then used cell-biologic and proteomic-based approaches to explore the effect of VEPTP on ANGPT2–TIE2 activity. Our results show that VEPTP functions as a molecular "rheostat," modulating receptor sensitivity to enable discrimination between ANGPT ligands, and provide a molecular mechanism to explain the opposing roles of ANGPT2 in blood and lymphatic vasculature.

Results

ANGPT2–TIE2 Signaling Is Essential for Embryonic Lymphangiogenesis. To identify the molecular basis of the differential functions of ANGPT2 in LECs and BECs, we characterized the role of ANGPT2–TIE2 signaling in lymphatic development, where ANGPT2 has a well-defined agonistic role (22–24). As expected, whole-body *Tie2* deletion from conception using the *Rosa26rtTA*; *tetOCre* bitransgenic system in mice harboring a *Tie2* conditional by inversion (COIN) allele ($Tie2^{WB\Delta E0}$) resulted in embryonic lethality between E9.5 and E10.5 (17, 18). However, embryos induced at E12.5 were found to survive until late gestation, allowing analysis of the lymphatic vasculature. At E16.5, all embryos were found alive. However, subcutaneous edema was observed in $Tie2^{WB\Delta E12.5}$ knockout embryos, which was never observed in *Tie2* wild-type or heterozygous controls (Fig. 1*A*). This result was recapitulated in lymphatic-specific *Tie2* knockouts generated using *Prox1*CreER ($Tie2^{Lym\Delta E10.5}$ mice), indicating that the edema was lymphatic in origin (Fig. 1*B*).

Compared with control littermates, immunostaining revealed a paucity of $PROX1^+$ lymphatic vessels in the dorsal skin of $Tie2^{Lym\Delta E10.5}$ knockout mice at E14.5, confirming the importance of lymphatic-expressed TIE2 in lymphangiogenesis (Fig. 1*C*). Interestingly, unlike the phenotype observed in lymphatic-specific knockout embryos, dorsal skin lymphatics of whole-body knockouts were enlarged compared with littermate controls (Fig. S1*A*). This enlargement could be due to the combined deleterious effects of TIE2 deletion on BECs and LECs.

To determine the role for each ANGPT ligand in dermal lymphangiogenesis, we compared the phenotypes of either single or combined deletion of *Angpt1* and *Angpt2* genes with *Tie2* conditional knockout mice. Unlike the marked edema of $Tie2^{WB\Delta E12.5}$ embryos, single *Angpt1* or *Angpt2* whole-body knockout embryos induced at E12.5 had no apparent edema when dissected at E16.5 (Fig. 1*D* and Fig. S1*B*). The fact that loss of the TIE2 receptor led to a more severe phenotype than loss of either ligand alone suggested compensation or cooperative roles of the two ANGPT ligands. To test this possibility, we generated compound mutants lacking both *Angpt1* and *Angpt2* from E12.5 onward. This simultaneous loss of ANGPT1 and ANGPT2 expression ($A1;A2^{WB\Delta E12.5}$) recapitulated the phenotype of $Tie2^{WB\Delta E12.5}$ mice (Fig. 1*D* and Fig. S1*B*) and embryos exhibited marked edema.

ANGPT2–TIE2 Signaling Is Essential for Mesenteric Lymphatic Development. *Angpt2* has a well-described role in the mesenteric and intestinal lymphatic vasculature. Because *Tie2* whole-body or lymphatic knockout embryos induced at E12.5 were not viable, we tested whole-body deletion at a range of time points (22–24). Deletion at E13.5 or later resulted in viable mutant offspring. $Tie2^{WB\Delta E13.5}$ knockout pups exhibited chylous ascites

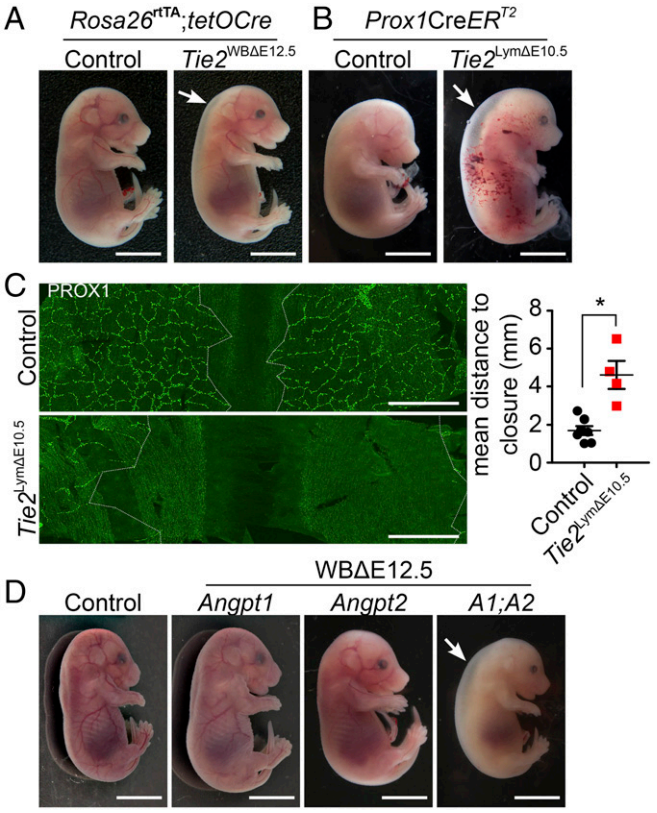

Fig. 1. ANGPT2–TIE2 signaling is essential for dermal lymphatic development. (*A* and *B*) Subcutaneous edema was observed following genetic deletion of *Tie2*. Gross view of mice with each genotype at E16.5 is shown. White arrows indicate subcutaneous skin edema. In *A*, doxycycline was given to pregnant dam from E12.5 to E16.5. In *B*, Tamoxifen was given from E10.5. (*C*) Lymphatic specific deletion of *Tie2* results in sparse lymphatic vessel development. Tamoxifen was injected from E10.5 and mice were dissected at E14.5. Whole-mount immunofluorescent staining of embryonic skin with the antibody against PROX1 is shown. $*P < 0.05$ vs. control. Two-tailed Student's *t* test was used. (*D*) Subcutaneous edema was observed following genetic deletion of both *Angpt1* and *Angpt2* but not either alone. (Scale bars: 5 mm in *A*, *B*, and *D*; 250 μm in *C*.)

with severely disturbed lymphatic vessel morphology, indicating defects of mesenteric lymphatic function (Fig. S2). We then examined the role of each ANGPT ligand in this TIE2-mediated developmental process. Whole-body *Angpt1* knockout pups induced at E13.5 had no apparent phenotype, but chylous ascites were observed in mice lacking *Angpt2* alone or both *Angpt1* and *Angpt2* (Fig. S2*B*). Deletion of *Tie2* after E15.5 did not result in overt chylous ascites (Fig. S2*A*), although a reduced number of lymphatic valves was observed in the mesentery (Fig. S3).

To better understand the role of ANGPT2 in mesenteric lymphatic development, we utilized a cell-type–specific approach to delete *Angpt2* in endothelial cells (24). In contrast to the well-developed lymphatic vessels in control mice, endothelial deletion of *Angpt2* using a lymphatic-expressed *Lyve1*-Cre ($Angpt2^{\Delta Lyve1Cre}$) resulted in severely disturbed lymphatic vessel morphology with leakage of chyle, phenocopying the $Tie2^{WB\Delta E13.5}$ knockout (Fig. S2*D*). Collectively, these results demonstrate a requirement for ANGPT2–TIE2 signaling in mesenteric lymphatic development.

A Regulatory Phosphatase for TIE2, VEPTP, Is Absent from Lymphatic Endothelium. TIE1 and VEPTP are both known to modulate activation status of TIE2 and are expressed in BECs (1–4). However, while TIE1 is expressed in LECs in vivo where it is required for lymphatic development (26, 31), VEPTP expression

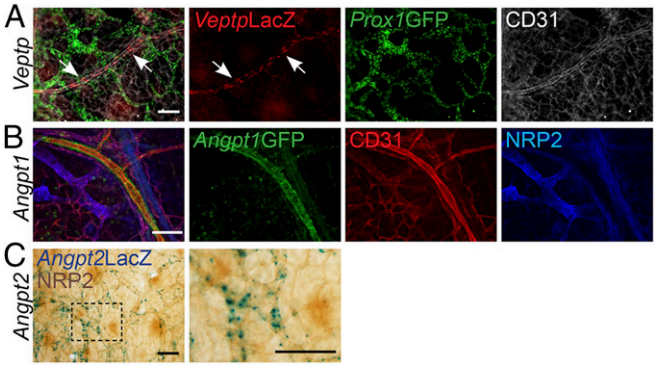

Fig. 2. Developing dermal lymphatic vessels lack VEPTP expression. (*A–C*) Expression pattern of *Veptp, Angpt1*, and *Angpt2* in embryonic dermis at E15.5. Knockin reporter mouse lines were used to detect expression. Whole-mount immunofluorescent imaging of embryonic skin dermis was performed with the antibody against β-gal [GFP, CD31, and neuropilin-2 (NRP2)]. Transgenic reporter mice harboring both *Veptp*$^{Lacz/+}$ and Tg-*Prox1-GFP* were analyzed in *A*. In *C*, X-gal staining of whole-mount skin of *Angpt2* $^{Lacz/+}$ mice was performed and counterstained with NRP2, a marker of the lymphatic endothelium. White arrows in *A* indicate VEPTP-expressing small artery in *A*. *C*, *Right* shows higher magnification of dotted box in *C*, *Left*. (Scale bars: 100 μm.)

in LECs in vivo has not been reported, although it has been reported in cultured human dermal LECs (32, 33). Using a *Veptp* reporter mouse line (30), we determined the expression pattern of the phosphatase. Both β-galactosidase expression and its activity were strongly detected in BECs, but not in PROX1 or LYVE1^{+} lymphatics of the embryonic dorsal skin, neonatal mesentery, adult ear dermis, or adult ocular limbus (Fig. 2*A* and Fig. S4). Interestingly, ANGPT1-producing cells are closely associated with CD31^{+} blood vessels but not with NRP2^{+} lymphatic vessels in embryonic skin (Fig. 2*B*). In contrast, *Angpt2* is expressed in NRP2^{+} dermal lymphatic vessels (Fig. 2*C*).

VEPTP Abrogates ANGPT2 Agonistic Activity on TIE2. Given the striking difference in expression pattern of VEPTP in blood vs. lymphatic vasculature in mice in vivo and its negative regulatory role on TIE2, we reasoned that VEPTP might block ANGPT2 agonistic function in BECs. To test this hypothesis, we characterized the interaction of TIE2 and VEPTP in a heterologous cell model using HEK293 cells, where endogenous expression of both proteins was absent. In cotransfection experiments, VEPTP effectively reduced autophosphorylation of TIE2. Phosphorylation was restored by treatment with a small-molecule inhibitor of VEPTP, AKB-9785, or with recombinant human (rHu) ANGPT1 (Fig. 3 *A* and *B*). VEPTP also reduced the phosphorylation of a TIE2 gain-of-function mutant (R849W) identified in patients with hereditary venous malformations (16), confirming its high enzymatic activity (Fig. S5*A*). TIE2 and VEPTP form a stable complex when transfected in cells and they reciprocally regulate each other, as evidenced by the TIE2-dependent phosphorylation of catalytically inactive VEPTP (Fig. S5 *B* and *C*).

Receptor tyrosine kinases signal through trans- and autophosphorylation of tyrosine residues (34). To better understand the regulation of TIE2 by VEPTP and TIE1, we performed phosphoproteomic analysis of the full-length TIE2 and TIE1 receptors in cells. Overall, mass spectrometry detected peptide fragments covering all but Y1024 of the 19 intracellular tyrosine residues on TIE2 (Fig. 3*C*). Among the 18 tyrosine residues, 13 were phosphorylated at varying levels, including the C-terminal Y1102 and Y1108 that are known to recruit downstream signaling adaptors, such as p85 of PI3 kinase and DokR (35–37). Juxta-membrane Y816, which has been reported to recruit Shp2 and Grb14 for signaling, was also phosphorylated (35). Next, we tested how VEPTP coexpression modulates baseline TIE2 phosphorylation levels on individual tyrosine sites. We observed marked reduction of all phosphorylation in the presence of VEPTP, highlighting the broad impact of this phosphatase to overall TIE2 signal strength (Fig. 3*C*).

TIE1, a homolog of TIE2, is an orphan receptor tyrosine kinase with no known ligand (1, 3). TIE1 has been shown to interact with TIE2 and its importance both in lymphangiogenesis

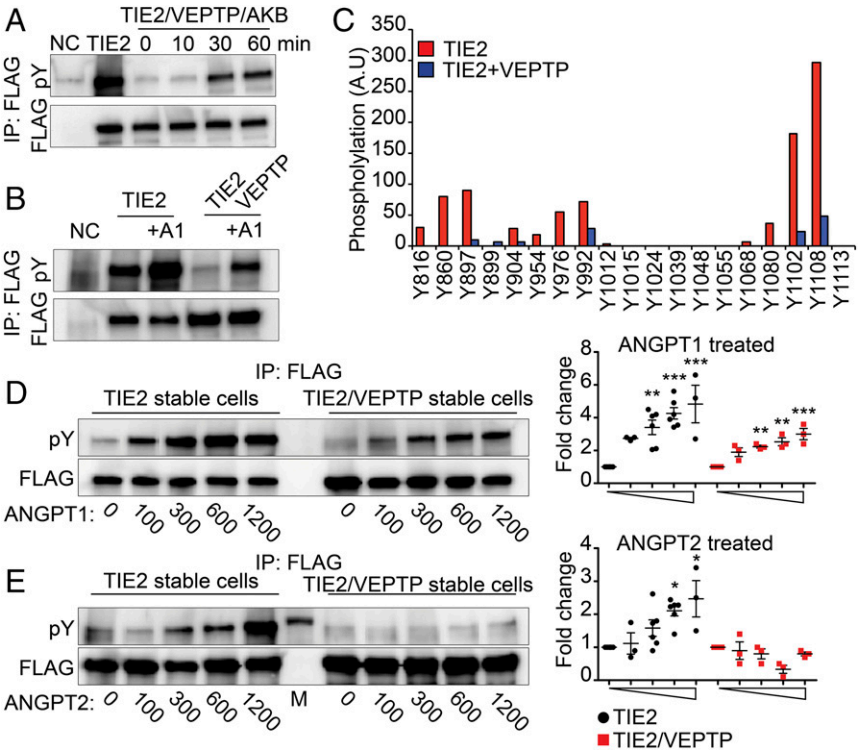

Fig. 3. VEPTP abrogates agonistic activity of ANGPT2 on TIE2 receptor. (*A* and *B*) TIE2 phosphorylation in HEK293 cells expressing either TIE2-FLAG alone or coexpressed with VEPTP-GFP. Cells were incubated with rHuANGPT1 (A1, 200 ng/mL) or a small-molecule inhibitor of VEPTP (AKB-9785, 15 μM). Phosphorylation of TIE2 was tested by immunoblotting against phospho-tyrosine (pY) following immunoprecipitation using anti-FLAG beads. (*C*) Phospho-mapping of TIE2-FLAG protein. pY residue was determined using mass spectrometry. Averaged intensity of two independent experiments with duplicated detection is shown. (*D* and *E*) TIE2 phosphorylation in HEK293 cells with stable expression of TIE2-FLAG alone or together with VEPTP-GFP (clone#3). The cells were incubated with either rHuANGPT1 (0–1,200 ng/mL) or rHuANGPT2 (0–1,200 ng/mL). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. negative control. One-way ANOVA with Tukey–Kramer correction was used. Full-length blot images are available in Fig. S8.

and angiogenesis has been well-characterized (26, 31, 38, 39). Recently, extracellular cleavage of TIE1 has been shown to play a role in ANGPT2-mediated TIE2 antagonism in BECs during inflammation (40). Our heterologous expression system showed that TIE1 maintained low levels of autophophosphorylation compared with TIE2 (Fig. S6*A*). Phosphoproteomic analysis of TIE1, either with or without TIE2, revealed that TIE1 phosphorylation is induced by TIE2 coexpression (Fig. S6*B*). Conversely, when a TIE2 kinase dead mutant protein was coexpressed with wild-type TIE1, the kinase-dead TIE2 became robustly phosphorylated (Fig. S6*C*), indicating reciprocal cross-talk between these kinases.

Ligand-induced TIE2 activation was studied using stable cell-lines expressing either TIE2-FLAG alone or together with VEPTP-GFP in HEK293 cells (Fig. S7*A*). Treatment with rHuANGPT1 and rHuANGPT2 increased TIE2 phosphorylation in TIE2-expressing cells in a dose-dependent fashion (Fig. 3 *D* and *E*). However, in the presence of VEPTP, only rHuANGPT1 was found to activate TIE2, while treatment with rHuANGPT2 had no effect (Fig. 3 *D* and *E*).

VEPTP Inhibition Restores ANGPT2 Agonistic Activity on TIE2. In a variety of disease conditions, circulating ANGPT2 levels increase, leading to elevated ANGPT2:ANGPT1 ratios (2, 7, 19, 41). We wondered if VEPTP inhibition might enable ANGPT2 to become a TIE2 agonist in BECs as seen in LECs. VEPTP inhibition alone or in combination with rHuANGPT2 treatment increased TIE2 phosphorylation in TIE2/VEPTP-expressing stable cells, whereas rHuANGPT2 alone did not (Figs. 3*E* and 4*A*). To determine if ANGPT2 enhances TIE2 signaling above the effect of VEPTP inhibition alone, we tested the downstream signaling activity in response to either ANGPT2 or VEPTP inhibition alone or in combination in primary and transformed endothelial cell-lines. Human umbilical vein endothelial cells (HUVECs) and EA.Hy926 cells express abundant TIE2 and VEPTP (Fig. S7*B*). Treatment with rHuANGPT1, but not with rHuANGPT2, increased pAKT levels in these cell lines (Fig. 4*B* and Fig. S7*C*). In contrast, rHuANGPT2 increased pAKT abundance in HUVECs, which were also treated with VEPTP inhibitor (Fig. 4*C* and Fig. S7*D*). Importantly, the level of AKT phosphorylation was greater in cells treated with both rHuANGPT2 and VEPTP inhibitor than with VEPTP inhibitor alone. Further downstream, ANGPT1-mediated increases in pAKT signaling are reported to cause forkhead box O1 (FOXO1) phosphorylation, leading to its nuclear exclusion (40, 41). This finding was reproduced in our model, where rHuANGPT1 induced nuclear FOXO1 protein to translocate to the cytoplasm, while rHuANGPT2 did not (Fig. 4*D*). However, consistent with our pAKT findings, treatment with VEPTP inhibitor alone or VEPTP inhibitor in combination with rHuANGPT2 also markedly reduced nuclear accumulation of FOXO1 (Fig. 4*D*).

ANGPT1 reinforces vascular junctions and stabilizes blood vasculature by initiating TIE2 phosphorylation and activating downstream signaling networks, but also by physically bridging TIE2 receptors between juxtaposed cells (42, 43). Immunocytochemical analyses revealed TIE2-FLAG protein localized at cellular junctions following stimulation with either rHuANGPT1 or rHuANGPT2, but not VEPTP inhibitor, as previously reported (28, 42). This ANGPT2-induced cellular junctional localization was not affected by VEPTP inhibition (Fig. S7*E*). Collectively, our results have demonstrated that VEPTP inhibition in conjunction with ANGPT2 stimulation activates TIE2 signaling and drives translocation of TIE2 to the cell junctions in BECs, mimicking the actions of ANGPT1.

Discussion

The ANGPT–TIE pathway is comprised of two receptor tyrosine kinases (TIE1 and TIE2), three ligands (ANGPT1, -2, -3/4), and one phosphatase (VEPTP), which serves as a negative regulator of TIE2 activation (1–4). While previous knockout mouse studies have demonstrated a role for ANGPT2 in lymphatic development, to our knowledge this report of a requirement for TIE2 signaling in lymphatic development, demonstrating ANGPT2 activation of TIE2 occurs in LECs in vivo, is unique.

Angiogenesis and lymphangiogenesis share several fundamental signaling cascades, including growth factor/receptor tyrosine kinase pathways needed to establish and remodel the vascular plexus (1–4). However, while many growth factors have similar effects on LECs and BECs (4), ANGPT2 has opposing effects on TIE2 signaling in cultured BECs vs. LECs (1, 3, 4, 12, 21). Many studies have been performed to identify the molecular basis of the context-dependent functions of ANGPT2 (14), as

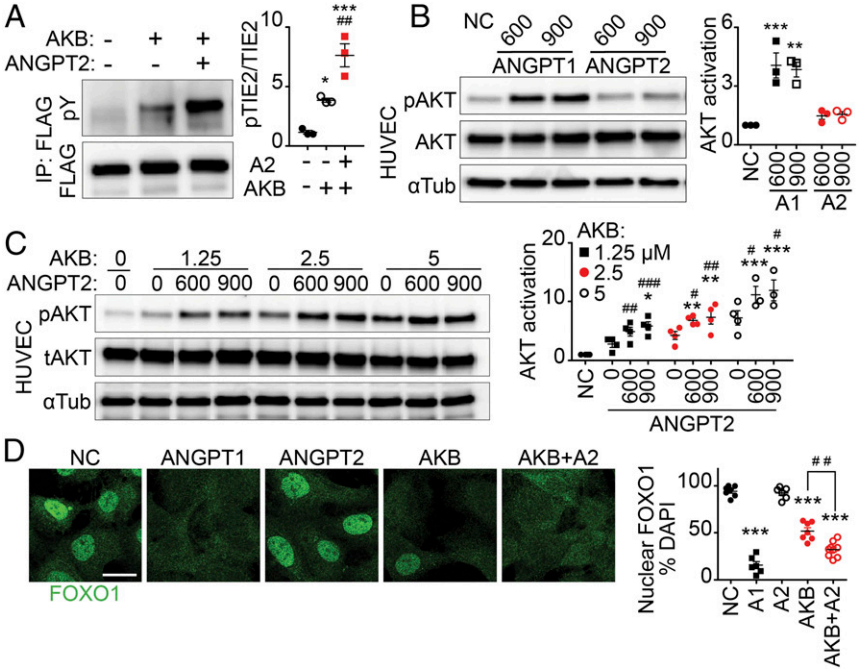

Fig. 4. VEPTP inhibition confers ANGPT2 agonistic function in blood endothelial cells. (*A*) TIE2 phosphorylation in TIE2-FLAG/VEPTP-GFP stable cells. Cells were incubated with rHuANGPT2 (600 ng/mL) and/or VEPTP inhibitor (AKB, AKB-9785 5 μM). $^{*}P <$ 0.05 and $^{***}P < 0.001$ vs. negative control. $^{\#\#}P <$ 0.01 vs. AKB alone. (*B* and *C*) AKT phosphorylation after stimulation by ANGPT ligands with/without VEPTP inhibitor (AKB). Note the dose-dependent increase of pAKT abundance by AKB-9785 and synergistic up-regulation of pAKT abundance with ANGPT2 cotreatments in *C*. AKB (0–5 μM); ANGPT1 (0–900 ng/mL); ANGPT2 (0–900 ng/mL). αTub, α-tubulin. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P <$ 0.001 vs. NC (negative control). $^{\#}P < 0.05$, $^{\#\#}P <$ 0.01, and $^{\#\#\#}P < 0.001$ vs. AKB alone. (*D*) Expression pattern of FOXO1 in EA.hy926 cells. The cells were treated with ANGPT ligands (600 ng/mL; A2, rHuANGPT2) and/or VEPTP inhibitor (AKB, 10 μM). Nuclear accumulation of FOXO1 protein was quantified in proportion to total $DAPI^{+}$ nuclear number. $^{***}P < 0.001$ vs. NC (negative control), $^{\#\#}P < 0.01$ vs. AKB. (Scale bar, 10 μm.) One-way ANOVA with Tukey–Kramer correction or Dunnett's correction was used. Full-length blot images are available in Fig. S8.

limited mechanistic understanding of this ligand remains an obstacle to therapeutic development targeting the ANGPT–TIE2 pathway. Outside of the lymphatic endothelium, researchers have identified several conditions in which ANGPT2 can act as an agonist, including (*i*) high ANGPT2 concentration in vitro (44), (*ii*) in stressed endothelial cells where FOXO1 nuclear accumulation is reduced (45), and (*iii*) the presence of functional TIE1 protein in cells where TIE2 is expressed (40). Additionally, ANGPT2 exerts proangiogenic/vascular-destabilizing signals through integrin-mediated pathways (46, 47). We propose a simple new model, where TIE2 receptor sensitivity in BECs and LECs is established by the presence or absence of VEPTP (Fig. 5).

Recent studies have reported crystal structure analysis of TIE2 and provide evidence that the differences in oligomerization, but not the receptor-binding domains of ANGPT ligands, are a major determinant of their potency (39, 48). Higher oligomerization status is required to cluster the TIE2 receptors *in cis* and elicit downstream signaling cascades due to the relatively large "physical" distance between them, as characterized by the wide angular conformational structure on cell membrane (39, 48). Lower oligomerization status is sufficient to interact with TIE2 receptors across endothelial cellular junctions *in trans* between juxtaposed cells (39, 48). Although ANGPT2 can form high-order oligomers, it is primarily observed as a dimer (49). In contrast, ANGPT1 is expressed mostly in high-order oligomers through intermolecular disulfide bridges, giving ANGPT1 a stronger TIE2 clustering ability (49). Furthermore, chimeric fusion protein analyses showed both ANGPT1 and ANGPT2 receptor-binding domains have similar TIE2 activating functions when artificially multimerized, emphasizing the importance of oligomerization status (50). Consistent with the model proposed by Leppänen et al. (39), we show that ANGPT2 can bridge the TIE2 receptor *in trans*, as demonstrated by movement of TIE2 to interendothelial junctions upon ligand exposure, but cannot activate downstream signaling in HUVECs that require clustering TIE2 receptors *in cis*. We posit that the low availability of higher-order ANGPT2 oligomers necessitates a highly responsive cellular status, such as that provided by the absence of VEPTP, to efficiently activate TIE2 signaling.

The ability of phosphatases to set response thresholds for external signals has been described for signaling through the T cell antigen receptor (TCR), where phosphatases set the threshold for discrimination between self/weak antigen and strong agonist (51). For example, PTPN22 limits the downstream signal from TCR stimulated with a weak agonist, but allows full activation by strong antigens (52). In a similar manner, we propose that VEPTP limits ANGPT2-mediated TIE2 phosphorylation and downstream signaling by setting a high threshold, but allows TIE2 to be activated by the strong agonist, ANGPT1. The divergent expression pattern of VEPTP in blood vs. lymphatic endothelium explains how it mediates context-dependent functions of ANGPT2. In contrast, other known modulators of TIE2 receptor signaling, such as TIE1, are expressed in both LECs and BECs, making them less-likely candidates to explain endothelial cell-type–specific differences in ANGPT2 functions (26, 31, 53).

Reducing vascular leakage and increasing BEC stability through TIE2 activation is an exciting therapeutic strategy for vascular disease, and this is an area of intense research interest (1–4). Elevated levels of ANGPT2 are present in diseases characterized by vascular leak and inflammation, suggesting strategies to convert endogenous ANGPT2 into a TIE2 activator might be advantageous. Recent studies have reported that an ANGPT2-binding antibody, ABTAA, can induce multimerization of ANGPT2 without neutralizing it (20, 41). This antibody-clustered ANGPT2 mimics the effect of ANGPT1 in BECs, activating TIE2 and providing beneficial effects in preclinical models of sepsis and cancer (20, 41). In separate studies, VEPTP inhibition has also been shown to be beneficial in preclinical models of eye disease, sepsis, and stroke, reducing vascular leak and inflammation through TIE2 activation (28, 54, 55). While VEPTP inhibition results in enhanced ligand-independent phosphorylation of the TIE2 receptor, we propose that additional benefits of VEPTP inhibition might include conversion of elevated ANGPT2 in the injured area to an "ANGPT1-like" TIE2 agonist. In keeping with this hypothesis, pharmacological inhibition of VEPTP coupled with ANGPT2 activated TIE2-AKT signaling in a synergistic fashion.

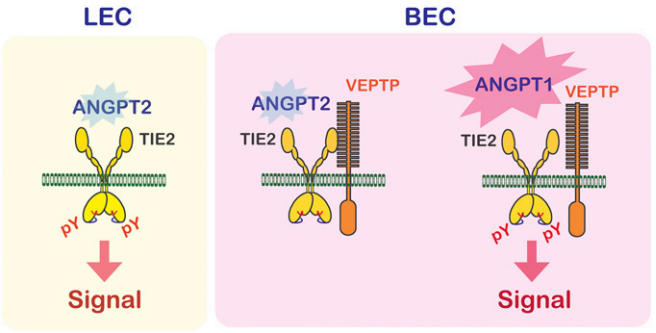

Fig. 5. VEPTP is a molecular "rheostat," modulating TIE2 receptor sensitivity to enable discrimination between ANGPT ligands. Schematic model showing the importance of VEPTP as a molecular rheostat, setting the threshold for TIE2 responsiveness to each ANGPT ligand. The absence of VEPTP in LECs lowers the threshold for TIE2 activation and allows ANGPT2 to activate TIE2-mediated downstream signaling cascades. However, ANGPT1 is required to activate TIE2 signaling in the BECs, in which VEPTP sets the higher threshold for TIE2 activation. VEPTP inhibition may lower the threshold in BECs and turns BECs into LEC-like ANGPT2-responsive cells in inflammatory diseases, thereby allowing ANGPT2 to activate TIE2-mediated vascular stabilizing signal.

In summary, we have shown that LECs lack VEPTP, conferring a TIE2 agonistic function on ANGPT2. Conversely, BECs express high levels of VEPTP, which raises the activation threshold of TIE2 and prevents activation by the weak agonist ANGPT2. The data support a model in which VEPTP serves as a molecular rheostat for TIE2 receptor sensitivity and confers a cell-type–specific function on ANGPT2. VEPTP inhibition is an attractive therapeutic target to promote vascular health through direct activation of the TIE2 receptor and conversion of ANGPT2 from an antagonist to an agonistic ligand (Fig. 5).

Materials and Methods

Animals. The mouse lines used for our study have been previously described (7, 17, 18, 24, 30, 56–58). Whole-body timed deletion of target genes was achieved by using a bigenic *Rosa26rtTA;tetOCre* system, as previously described (7, 17, 18). The transgenic mouse lines were maintained on a mixed background due to the large number of transgenes required. However, littermate controls were used for all phenotype analyses. Full details of mouse analysis are described in *SI Materials and Methods*.

Cell Culture Experiments and Phospho-Proteomics. The cells were cultured with standard methods and stimulated with rHuANGPT1 (R&D Systems), rHuANGPT2 (R&D Systems), and AKB-9785 (a VEPTP inhibitor) (54) for 30 min at 37 °C unless otherwise mentioned. For phosoho-mapping analysis, HEK293 cells were transfected with plasmid vectors for expressing TIE2-FLAG, TIE1-FLAG, and VEPTP-GFP, either alone or in combination. The proteins in corresponding SDS gel pieces were digested with trypsin and chymotrypsin, and digested peptides were analyzed with LC-MS/MS. Abundance of phophorlyation sites were semiquantitatively compared across the samples using spectral counting (number of peptide spectrum matching or PSM) (59). Full details of analysis are described in *SI Materials and Methods*.

Statistics and Reproducibility. Results are expressed as means ± SEM. Statistical analysis was carried out using two-tailed Student's *t* test or one-way ANOVA followed by Tukey–Kramer's test or Dunnett's correction for

multiple comparisons using GraphPad Prism software. A *P* value less than 0.05 was considered as statistically significant.

Study Approval. All animal experiments were approved by the Animal Care Committee of Mount Sinai Hospital, University of Toronto, Toronto and the Institutional Animal Care and Use Committee of the Center for Comparative Medicine at Northwestern University, Chicago.

ACKNOWLEDGMENTS. We thank Dr. Sean Morrison (University of Texas Southwestern Medical Center) for the generous gift of *Angpt1*^{GFP} mice; Dr. Guillermo Oliver and his team for providing critical comments and helpful suggestions on the manuscript; Venus Onay, Megan Kelly, Ronnie Anderson, and Anna Woo for their technical support; and Dr. Dhaval Nanavati (Northwestern University, Proteomics Core) for his support in proteomics analysis. Imaging was performed at the Northwestern University Center for Advanced Microscopy, supported by National Cancer Institute Cancer Center Support Grant P30 CA060553, awarded to the Robert H. Lurie Comprehensive Cancer Center. This study was funded by NIH Grants R01HL124120, NEI R01EY025799, and T32DK108738 (to S.E.Q.). T.S. is supported by NIH Grant R01HL124120 and fellowship grants from the Japan Society for the Promotion of Science and Mallinckrodt Pharmaceuticals.

1. Eklund L, Kangas J, Saharinen P (2017) Angiopoietin-Tie signalling in the cardiovascular and lymphatic systems. *Clin Sci (Lond)* 131:87–103.
2. Saharinen P, Eklund L, Alitalo K (2017) Therapeutic targeting of the angiopoietin-TIE pathway. *Nat Rev Drug Discov* 16:635–661.
3. Augustin HG, Koh GY, Thurston G, Alitalo K (2009) Control of vascular morphogenesis and homeostasis through the angiopoietin-Tie system. *Nat Rev Mol Cell Biol* 10: 165–177.
4. Jeltsch M, Leppänen VM, Saharinen P, Alitalo K (2013) Receptor tyrosine kinase-mediated angiogenesis. *Cold Spring Harb Perspect Biol* 5:a009183.
5. Fiedler U, et al. (2006) Angiopoietin-2 sensitizes endothelial cells to TNF-alpha and has a crucial role in the induction of inflammation. *Nat Med* 12:235–239.
6. Gavard J, Patel V, Gutkind JS (2008) Angiopoietin-1 prevents VEGF-induced endothelial permeability by sequestering Src through mDia. *Dev Cell* 14:25–36.
7. Jeansson M, et al. (2011) Angiopoietin-1 is essential in mouse vasculature during development and in response to injury. *J Clin Invest* 121:2278–2289.
8. Sato TN, Qin Y, Kozak CA, Audus KL (1993) Tie-1 and Tie-2 define another class of putative receptor tyrosine kinase genes expressed in early embryonic vascular system. *Proc Natl Acad Sci USA* 90:9355–9358.
9. Sato TN, et al. (1995) Distinct roles of the receptor tyrosine kinases Tie-1 and Tie-2 in blood vessel formation. *Nature* 376:70–74.
10. Dumont DJ, et al. (1994) Dominant-negative and targeted null mutations in the endothelial receptor tyrosine kinase, tek, reveal a critical role in vasculogenesis of the embryo. *Genes Dev* 8:1897–1909.
11. Suri C, et al. (1996) Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. *Cell* 87:1171–1180.
12. Maisonpierre PC, et al. (1997) Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. *Science* 277:55–60.
13. Barton WA, et al. (2006) Crystal structures of the Tie2 receptor ectodomain and the angiopoietin-2-Tie2 complex. *Nat Struct Mol Biol* 13:524–532.
14. Thurston G, Daly C (2012) The complex role of angiopoietin-2 in the angiopoietin-tie signaling pathway. *Cold Spring Harb Perspect Med* 2:a006550.
15. Fiedler U, et al. (2004) The Tie-2 ligand angiopoietin-2 is stored in and rapidly released upon stimulation from endothelial cell Weibel-Palade bodies. *Blood* 103:4150–4156.
16. Vikkula M, et al. (1996) Vascular dysmorphogenesis caused by an activating mutation in the receptor tyrosine kinase TIE2. *Cell* 87:1181–1190.
17. Thomson BR, et al. (2014) A lymphatic defect causes ocular hypertension and glaucoma in mice. *J Clin Invest* 124:4320–4324.
18. Souma T, et al. (2016) Angiopoietin receptor TEK mutations underlie primary congenital glaucoma with variable expressivity. *J Clin Invest* 126:2575–2587.
19. Parikh SM, et al. (2006) Excess circulating angiopoietin-2 may contribute to pulmonary vascular leak in sepsis in humans. *PLoS Med* 3:e46.
20. Park JS, et al. (2016) Normalization of tumor vessels by Tie2 activation and Ang2 inhibition enhances drug delivery and produces a favorable tumor microenvironment. *Cancer Cell* 30:953–967.
21. Nguyen VP, et al. (2007) Differential response of lymphatic, venous and arterial endothelial cells to angiopoietin-1 and angiopoietin-2. *BMC Cell Biol* 8:10.
22. Dellinger M, et al. (2008) Defective remodeling and maturation of the lymphatic vasculature in Angiopoietin-2 deficient mice. *Dev Biol* 319:309–320.
23. Zheng W, et al. (2014) Angiopoietin 2 regulates the transformation and integrity of lymphatic endothelial cell junctions. *Genes Dev* 28:1592–1603.
24. Gale NW, et al. (2002) Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. *Dev Cell* 3: 411–423.
25. Chu M, et al. (2016) Angiopoietin receptor Tie2 is required for vein specification and maintenance via regulating COUP-TFII. *eLife* 5:e21032.
26. Shen B, et al. (2014) Genetic dissection of Tie pathway in mouse lymphatic maturation and valve development. *Arterioscler Thromb Vasc Biol* 34:1221–1230.
27. Fachinger G, Deutsch U, Risau W (1999) Functional interaction of vascular endothelial-protein-tyrosine phosphatase with the angiopoietin receptor Tie-2. *Oncogene* 18: 5948–5953.
28. Frye M, et al. (2015) Interfering with VE-PTP stabilizes endothelial junctions in vivo via Tie-2 in the absence of VE-cadherin. *J Exp Med* 212:2267–2287.
29. Dominguez MG, et al. (2007) Vascular endothelial tyrosine phosphatase (VE-PTP)-null mice undergo vasculogenesis but die embryonically because of defects in angiogenesis. *Proc Natl Acad Sci USA* 104:3243–3248.
30. Bäumer S, et al. (2006) Vascular endothelial cell-specific phosphotyrosine phosphatase (VE-PTP) activity is required for blood vessel development. *Blood* 107:4754–4762.
31. D'Amico G, et al. (2010) Loss of endothelial Tie1 receptor impairs lymphatic vessel development-brief report. *Arterioscler Thromb Vasc Biol* 30:207–209.
32. Deng Y, Zhang X, Simons M (2015) Molecular controls of lymphatic VEGFR3 signaling. *Arterioscler Thromb Vasc Biol* 35:421–429.
33. Klein S, et al.; FANTOM consortium (2016) DeepCAGE transcriptomics identify HOXD10 as a transcription factor regulating lymphatic endothelial responses to VEGF-C. *J Cell Sci* 129:2573–2585.
34. Lemmon MA, Schlessinger J (2010) Cell signaling by receptor tyrosine kinases. *Cell* 141:1117–1134.
35. Jones N, et al. (1999) Identification of Tek/Tie2 binding partners. Binding to a multifunctional docking site mediates cell survival and migration. *J Biol Chem* 274: 30896–30905.
36. Kontos CD, et al. (1998) Tyrosine 1101 of Tie2 is the major site of association of p85 and is required for activation of phosphatidylinositol 3-kinase and Akt. *Mol Cell Biol* 18:4131–4140.
37. Jones N, et al. (2003) A unique autophosphorylation site on Tie2/Tek mediates Dok-R phosphotyrosine binding domain binding and function. *Mol Cell Biol* 23:2658–2668.
38. D'Amico G, et al. (2014) Tie1 deletion inhibits tumor growth and improves angiopoietin antagonist therapy. *J Clin Invest* 124:824–834.
39. Leppänen VM, Saharinen P, Alitalo K (2017) Structural basis of Tie2 activation and Tie2/Tie1 heterodimerization. *Proc Natl Acad Sci USA* 114:4376–4381.
40. Korhonen EA, et al. (2016) Tie1 controls angiopoietin function in vascular remodeling and inflammation. *J Clin Invest* 126:3495–3510.
41. Han S, et al. (2016) Amelioration of sepsis by TIE2 activation-induced vascular protection. *Sci Transl Med* 8:335ra55.
42. Saharinen P, et al. (2008) Angiopoietins assemble distinct Tie2 signalling complexes in endothelial cell-cell and cell-matrix contacts. *Nat Cell Biol* 10:527–537.
43. Fukuhara S, et al. (2008) Differential function of Tie2 at cell-cell contacts and cell-substratum contacts regulated by angiopoietin-1. *Nat Cell Biol* 10:513–526.
44. Kim I, et al. (2000) Angiopoietin-2 at high concentration can enhance endothelial cell survival through the phosphatidylinositol 3′-kinase/Akt signal transduction pathway. *Oncogene* 19:4549–4552.
45. Daly C, et al. (2006) Angiopoietin-2 functions as an autocrine protective factor in stressed endothelial cells. *Proc Natl Acad Sci USA* 103:15491–15496.
46. Hakanpaa L, et al. (2015) Endothelial destabilization by angiopoietin-2 via integrin β1 activation. *Nat Commun* 6:5962.
47. Felcht M, et al. (2012) Angiopoietin-2 differentially regulates angiogenesis through TIE2 and integrin signaling. *J Clin Invest* 122:1991–2005.
48. Moore JO, Lemmon MA, Ferguson KM (2017) Dimerization of Tie2 mediated by its membrane-proximal FNIII domains. *Proc Natl Acad Sci USA* 114:4382–4387.
49. Kim KT, et al. (2005) Oligomerization and multimerization are critical for angiopoietin-1 to bind and phosphorylate Tie2. *J Biol Chem* 280:20126–20131.
50. Kim HZ, Jung K, Kim HM, Cheng Y, Koh GY (2009) A designed angiopoietin-2 variant, pentameric COMP-Ang2, strongly activates Tie2 receptor and stimulates angiogenesis. *Biochim Biophys Acta* 1793:772–780.
51. Stanford SM, Rapini N, Bottini N (2012) Regulation of TCR signalling by tyrosine phosphatases: From immune homeostasis to autoimmunity. *Immunology* 137:1–19.
52. Salmond RJ, Brownlie RJ, Morrison VL, Zamoyska R (2014) The tyrosine phosphatase PTPN22 discriminates weak self peptides from strong agonist TCR signals. *Nat Immunol* 15:875–883.
53. Savant S, et al. (2015) The orphan receptor Tie1 controls angiogenesis and vascular remodeling by differentially regulating Tie2 in tip and stalk cells. *Cell Rep* 12: 1761–1773.
54. Gurnik S, et al. (2016) Angiopoietin-2-induced blood-brain barrier compromise and increased stroke size are rescued by VE-PTP-dependent restoration of Tie2 signaling. *Acta Neuropathol* 131:753–773.
55. Shen J, et al. (2014) Targeting VE-PTP activates TIE2 and stabilizes the ocular vasculature. *J Clin Invest* 124:4564–4576.
56. Zhou BO, Ding L, Morrison SJ (2015) Hematopoietic stem and progenitor cells regulate the regeneration of their niche by secreting Angiopoietin-1. *eLife* 4:e05521.
57. Choi I, et al. (2011) Visualization of lymphatic vessels by Prox1-promoter directed GFP reporter in a bacterial artificial chromosome-based transgenic mouse. *Blood* 117: 362–365.
58. Pham TH, et al. (2010) Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *J Exp Med* 207:17–27.
59. Huttlin EL, et al. (2010) A tissue-specific atlas of mouse protein phosphorylation and expression. *Cell* 143:1174–1189.