Sec14l3 potentiates VEGFR2 signaling to regulate zebrafish vasculogenesis

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Vascular endothelial growth factor (VEGF) regulates vasculogenesis by using its tyrosine kinase receptors. However, little is known about whether Sec14-like phosphatidylinositol transfer proteins (PTP) are involved in this process. Here, we show that zebrafish sec14l3, one of the family members, specifically participates in artery and vein formation via regulating angioblasts and subsequent venous progenitors’ migration during vasculogenesis. Vascular defects caused by sec14l3 depletion are partially rescued by restoration of VEGFR2 signaling at the receptor or downstream effector level. Biochemical analyses show that Sec14l3/SEC14L2 physically bind to VEGFR2 and prevent it from dephosphorylation specifically at the Y¹¹⁷⁵ site by peri-membrane tyrosine phosphatase PTP1B, therefore potentiating VEGFR2 signaling activation. Meanwhile, Sec14l3 and SEC14L2 interact with RAB5A/4A and facilitate the formation of their GTP-bound states, which might be critical for VEGFR2 endocytic trafficking. Thus, we conclude that Sec14l3 controls vasculogenesis in zebrafish via the regulation of VEGFR2 activation.

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The vertebrate vasculature, as a tree-like tubular and highly dynamic plexus, extends into almost all tissues for a constant supply of nutrients and oxygen, or transport of metabolic wastes\textsuperscript{13-15}. The formation of a functional vascular system is essential for embryonic development, and its structural abnormalities always lead to pathological diseases\textsuperscript{33-34}. This systematic and hierarchical vascular system is achieved by two distinct mechanisms, vasculosogenesis (de novo assembly of vessels) and angiogenesis (modification and expansion of pre-existing vessels). In zebrafish, angioblasts derived from the lateral plate mesoderm eventually give rise to the first embryonic artery (dorsal aorta, DA) and vein (posterior cardinal vein, PCV) during vasculosogenesis, and then these vascular systems are rapidly expanded and remodeled during angiogenesis to consummate vessel networks, including the formation of intersegmental veins (ISV) by endothelial cell sprouting\textsuperscript{5-7}.

So far, a variety of signaling molecules and transcription factors have been implicated in the formation of the vertebrate vasculature via regulating endothelial cell proliferation, differentiation, migration, and apoptosis\textsuperscript{3-4,6}. Vascular endothelial growth factor (VEGF) signaling is considered as the most critical and pivotal one during embryonic vasculosogenesis as well as angiogenesis\textsuperscript{9,10}. After secretion, VEGF ligands bind in an overlapping pattern to three receptor tyrosine kinases (RTKs), known as VEGFR1/Flt-1, VEGFR2/Flik-1/KDR, and VEGFR3/Flt-4 on the plasma membrane, followed by receptor dimerization and autophosphorylation at particular tyrosine sites. Then, the phosphorylated receptors recruit interacting proteins and further trigger the activation of downstream cascades via PLC\textgamma

To achieve specific signal outputs with coordinated duration and amplitude, VEGFR2 signaling is strictly regulated at numerous levels, such as the receptor expression level, the availability, and affinities for binding its different ligands, the presence of co-receptors and repressor (tyrosine phosphatases), and so on\textsuperscript{15-17}. More importantly, the intracellular trafficking and endocytic kinetics of VEGFR2 also regulate the signal outputs significantly\textsuperscript{18-20}. Upon ligand binding, VEGFR2 is internalized mainly in a clathrin-dependent manner with the help of motor proteins and trafficked to Rab5-positive and EEA1-positive early endosomes. Unless VEGFR2 is dephosphorylated by the periliminal resident PTP1B and trafficked to Rab7-pathway to attenuate the signaling or recycled back to the plasma membrane via the fast (RAB4-dependent) or slow (RAB11-dependent) route for further potentiation\textsuperscript{19}. Although NRP1-synectin-myoVI and ephrinB2-DAB2-PAR3 complex have been demonstrated to promote endosome movements into cell\textsuperscript{22,23}, many events involved in VEGFR2 internalization and trafficking are still unclear. Full understanding of the endocytic VEGFR2 trafficking will further advance our understanding of the VEGF signaling and the consequential biological functions.

Sec14l3 proteins belong to phosphatidylinositol transfer proteins (PITPs), which were first described as transporters to potentiate phosphatidylinositol (PI) and phosphatidylcholine (PC) exchange between membranes in vitro\textsuperscript{24}. Because of the involvement of PI molecules in endocytic membrane trafficking, PITPs are also proposed to play a vital role in vesicle budding from the trans Golgi network (TGN) as well as in priming of exocytosis\textsuperscript{25-27}. Although it is reported that there is a crosstalk between PITPs with Wnt/Ca\textsuperscript{2+} and EGF signaling\textsuperscript{28-30}, whether PITPs integrate lipid metabolism with intracellular VEGF signaling has not been studied.

In this paper, we demonstrate that Sec14l3 regulates VEGF signaling at its receptor level and participates in vascular development of zebrafish embryos. Using morpholino knockdown in combination with CRISPR/Cas9 knockout system, we find that sec14l3 deficiency results in narrowed DA and PCV lumens. Mechanistically, Sec14l3 promotes RAB5A/4A activation to accelerate VEGFR2 internalization and recycling, thus preventing VEGFR2 from exposure to the phosfatase activity of PTP1B, which ultimately enhances the downstream VEGF signaling. It will be meaningful to interpret PITPs function in regulating VEGFR2 trafficking as well as in understanding VEGF signaling during vertebrate vasculature development.

Results
Sec14l3/SEC14L2 are enriched in the endothelial cells. Vascular system formation is important for the normal development of vertebrate embryos, as well as for the survival of adults\textsuperscript{4}. In order to identify critical genes required for zebrafish vasculature development, we isolated GFP-positive cell populations, by fluorescence-activated cell sorting (FACS), from Tg(fli1a:EGFP)\textsuperscript{1} embryos at 24 hpf, when the DA has separated from the PCV\texttextsuperscript{5,6}. RNA sequencing of the isolated cells showed that genes in vascular, hematopoietic and pharyngeal arch cells were highly expressed as reported previously (Supplementary data 1)\textsuperscript{1}. Of our interest is the PTP gene sec14l3. Whole-mount in situ hybridization (WISH) results showed that sec14l3 was spatially expressed in vasculature at 24 hpf (Fig. 1a). Transverse sectioning post WISH indicated that sec14l3 is expressed in both arterial and venous endothelial progenitor cells at 19 hpf, and thereafter it is restricted to the PCV compartment at 25 hpf (Fig. 1a). The expression of sec14l3 in endothelial cells is almost eliminated in etsrp/etrv2 mutants lacking trunk vasculature (Fig. 1b)\textsuperscript{31}, which confirms its vascular expression. Quantitative RT-PCR analysis indicated that sec14l3 expression level in GFP\textsuperscript{+} cells from Tg (fli1a:EGFP)\textsuperscript{1} embryos at 24 hpf is enriched by about 5 folds, which is comparable to that of kdr/vegfr2, a specific vascular marker gene (Fig. 1c).

To know whether the enrichment of sec14l3 in endothelial cells is conserved in mammals, we analyzed human SEC14L2/SEC14L3 expression in human umbilical vein endothelial cells (HUVECs). RT-PCR results showed that SEC14L2 transcripts exist while SEC14L3 does not (Supplementary Fig. 1a). Importantly, immunostaining data demonstrated that SEC14L2 protein locates in vasculature at 24 hpf (Fig. 1a). The expression of sec14l3 in endothelial cells is almost eliminated in etsrp/etrv2 mutants lacking trunk vasculature (Fig. 1b)\textsuperscript{31}, which confirms its vascular expression. Quantitative RT-PCR analysis indicated that sec14l3 expression level in GFP\textsuperscript{+} cells from Tg (fli1a:EGFP)\textsuperscript{1} embryos at 24 hpf is enriched by about 5 folds, which is comparable to that of kdr/vegfr2, a specific vascular marker gene (Fig. 1c).

Sec14l3/SEC14L2 are required for vascular formation. To investigate whether Sec14l3 is required for vasculature development during zebrafish embryogenesis, two antisense morpholino oligonucleotides (MO), sec14l3-tMO2 and sec14l3-sMO, were designed to knock down sec14l3 expression in vivo. sec14l3-tMO2 targets the 5’ untranslated region of sec14l3 mRNA to block its translation, while sec14l3-sMO targets the 2nd exon-intron junction to interrupt normal splicing of the primary transcript. Their effectiveness was confirmed by previous report\textsuperscript{30} and...
RT-PCR analysis (Supplementary Fig. 1b). Compared to control embryos injected with standard MO (std-MO), 5 ng sec14l3-tMO2 injection showed no obvious difference in morphology, with normal convergent and extension movements at gastrulation stages (Supplementary Fig. 1c). When analyzing vascular development, we found that knockdown of sec14l3 in Tg(kdrl:GFP)s843Tg embryos resulted in arterial-venous segregation and luminal expansion defects with compromised ISVs formation (Fig. 2a and Supplementary Fig. 2a). However, when sec14l3-tMO2 was injected in a p53 mutant background, ISVs sprouting defect was largely restored at 48 hpf (Fig. 2a and Supplementary Fig. 2b), which presumably relieves the general off-target effect of morpholinos33. Therefore, we conclude that sec14l3 specifically regulates DA and PCV formation during vasculogenesis.

To figure out sec14l3 functions in a maternal- or zygotic-dependent manner, we turned to use the splicing blocker sec14l3-sMO. Consistent with sec14l3-tMO2, sec14l3-sMO injection similarly inhibited the arterial-venous segregation at 23 hpf and luminal expansion at 25 hpf (Fig. 2b, c), suggesting that the vascular defects mainly arise from the loss of zygotic sec14l3. More importantly, the DA and PCV defects in sec14l3 morphants were restored by sec14l3 mRNA overexpression (Fig. 2b, c), indicative of specific effects.

To substantiate the knockdown effect, we also generated a sec14l3 mutant based on clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 technology, using a guide RNA targeting the 5th exon of sec14l3 gene in Tg(kdrl:GFP)s843Tg transgenic line (Supplementary Fig. 3a). The isolated mutant allele contains a 10-bp deletion near the target site, causing a premature stop codon and presumably a truncated protein only owning CRAL-TRIO domain (Supplementary Fig. 3a). Although there is no obvious difference between mutant and wild-type embryos in morphology (Supplementary Fig. 3b), sec14l3 transcripts are almost abolished in homozygous mutants.

Fig. 1 sec14l3/SEC14L2 are expressed in endothelial cells and co-localized with VEGFR2. a Expression pattern of sec14l3 examined by WISH. Trunk vascular expression of sec14l3 is shown at 24 hpf and the vertical line denotes the equivalent position of transverse sections in embryos at 19 hpf and 25 hpf. High-resolution views of the boxed regions are shown at right panels. Adjacent tissues are denoted. NT, neural tube; NC, notochord; DA, dorsal aorta; PCV, posterior cardinal vein; VC, vascular cord. Scale bars, 100 μm. b The absence of sec14l3 expression in the trunk vascular system of etsrp mutant embryos at 26 hpf. High-resolution views of the boxed regions are shown at right panels. Scale bars, 100 μm. c Quantitative RT-PCR of sec14l3 mRNA in both GFP+ cells and GFP- cells from Tg(flhia:EGFP)y1 transgenic embryos at 24 hpf. sec14l3 is highly enriched in GFP+ cells (blue bars) relative to negative cells (red bars), using β-actin as the internal control. kdrl/vegfr2 mRNA serves as a positive control for vascular endothelial cells. A two-tailed t-test was used for statistical analysis. *p < 0.05, the exact p-values in each figure are shown in the Source data file. d SEC14L2 is co-localized with VEGFR2 in HUVECs. Red and green represent anti-VEGFR2 antibody and anti-SEC14L2 antibody staining signal respectively; blue symbolizes DAPI stained nucleus. Regions in the boxes are enlarged in the right corner. Scale bars, 20 μm in the original pictures and 5 μm in the enlarged panels.
were used for experiments, 60 lentivirus and the complete tube number was scored per wound edges. Statistical data are shown (observation. The ratio of recovered wound width at a specific time point post-wounding to the initial wounding width was calculated. Dashed lines indicate wound edges. Statistical data are shown (n = 3). 1 sec14I3 knockdown inhibits in vitro tube formation. HUVECs were infected with control or sec14I3 shRNA were used for wound closure observation. The ratio of recovered wound width at a specific time point post-wounding to the initial wounding width was calculated. Dashed lines indicate wound edges. Statistical data are shown (n = 3). 1 sec14I3 knockdown inhibits in vitro tube formation. HUVECs were infected with shRNA-packed lentivirus and the complete tube number was scored per field at 24 h after plating. The statistical data are shown on the right (n = 3 independent experiments, 60 fields total). All statistical data are shown as mean ± SEM. One-way ANOVA tests were used for statistical analyses in c, e, and f-tests were used for h-i. **p < 0.01; ***p < 0.001; ns, not significant. Scale bars, 25 μm for a, 50 μm for b, d, g, and h-i, 100 μm for f. Source data are provided as a Source Data file

Fig. 2 sec14I3/SEC14L2 are required for vascular formation in vivo and in vitro. a sec14I3-tMO injection impairs arterial-venous segregation and luminal formation in zebrafish. Embryos of Tg(kdr:EGFP)843Tg;p53cre/+ (left panel) or Tg(kdr:EGFP)843Tg;p53cre−/− fish (right panel) were used. White and yellow brackets indicate DA and PCV respectively; red arrows indicate ISVs sprouting. The ratio in the right corner indicates the number of embryos with observed pattern/the total number of observed embryos. sec14I3-sMO mediated knockdown of sec14I3 causes trunk vascular defects. 0.5 ng sec14I3-sMO and 150 pg sec14I3 mRNA were co-injected for rescue experiment. c Statistical analyses of DA and PCV diameters at 25 hpf in b (n = 20 embryos).

d Vasculature defects in sec14I3 mutant embryos. Heterozygous mutants were intercrossed and embryos were harvested for vasculature observation and genotyping analysis individually. e Statistical analyses of DA and PCV diameters at 30 hpf in d. The total number of embryos in each genotype is indicated.

f sec14I3-sMO injection causes angioblast migration defects. 0.5 ng sec14I3-sMO and 100 pg sec14I3 mRNA were co-injected for rescue experiment. WISH using etsrp probe marks the medial (arrowhead) and lateral (arrow) progenitor populations simultaneously. g Ventral sprouting defects in sec14I3 mutant embryos. h SEC14L2 knockdown inhibits the in vitro wound closure process. HUVECs infected with control or SEC14L2 shRNA were used for wound closure observation. The ratio of recovered wound width at a specific time point post-wounding to the initial wounding width was calculated. Dashed lines indicate wound edges. Statistical data are shown (n = 3). i SEC14L2 knockdown inhibits in vitro tube formation. HUVECs were infected with shRNA-packed lentivirus and the complete tube number was scored per field at 24 h after plating. The statistical data are shown on the right (n = 3 independent experiments, 60 fields total). All statistical data are shown as mean ± SEM. One-way ANOVA tests were used for statistical analyses in c, e, and f-tests were used for h-i. **p < 0.01; ***p < 0.001; ns, not significant. Scale bars, 25 μm for a, 50 μm for b, d, g, and h-i, 100 μm for f. Source data are provided as a Source Data file

(Supplementary Fig. 3c). Compared with wild-type and heterozygous, homozygous embryos exhibit arterial-venous segregation and luminal expansion defects with nearly normal ISVs formation, distinct from impaired ISV length in sec14I3 morphants (Fig. 2d, e, and Supplementary Fig. 4). This is partially due to a non-specific effect of tMOs, as after injection of sec14I3-tMO2 in homozygotes, it could not cause further DA and PCV defects, but with further reduction of ISV length (Supplementary Fig. 4). Therefore, we conclude that sec14I3 is required for zebrafish DA and PCV formation.
Since it is reported that two distinct mechanisms could account for the DA and PCV formation in zebrafish embryos, we wonder what happens in our mutants. Using fl and eisrr as probes, the Wish result showed that in sec14l3 deficient embryos, the specification of angioblast is not affected at 8-somite stages (Supplementary Fig. 5), while its migration is significantly reduced at 14–16 somite stages, which could be rescued by the injection of sec14l3 mRNA (Fig. 2f). Meanwhile, with high-resolution imaging in Tg(kdr:GFP) larvae embryos, we found that the sprouting of venous progenitors from the vascular cord is also decreased from 21 to 23 hpf in sec14l3 deficient embryos (Fig. 2g and Supplementary Fig. 6). Therefore, we’d like to conclude that sec14l3 play important roles in zebrafish DA and PCV blood vessels formation via regulating angioblast migration.

Since Sec14l3/SEC14L2 promote VEGF-induced VEGFR2 activation. VEGF2 signaling plays vital roles during zebrafish vasculature, which could be motivated both by VEGF ligands and blood flow-induced fluid shear stress (FSS)\(^ {10,37}\). Given that Sec14l3 co-localizes with VEGFR2 in HUVECs (Fig. 1d), we hypothesized that Sec14l3 regulates VEGFR2 signaling. Since Sec14l3 exerts its function on angioblast migration during vasculogenesis before the onset of blood flow in vivo (Fig. 2f, g and Supplementary Fig. 6), we focused on the VEGF- but not FSS-induced VEGFR2 signaling transduction in vitro, and further investigated Sec14l3 knockdown effect in HUVECs on the downstream effectors of VEGF signaling to mimic the endogenous regulation. As shown in Fig. 3a, the addition of VEGF led to a rapid upregulation of p-ERK-1/2 (Thr202/Tyr204) and p-AKT (Ser473) in NC-shRNA transfected control cells. Strikingly, both of these effectors of VEGF signaling to mimic the endogenous regulation. As shown in Fig. 3a, the addition of VEGF led to a rapid upregulation of p-ERK-1/2 (Thr202/Tyr204) and p-AKT (Ser473) in NC-shRNA transfected control cells. Strikingly, both of these effectors of VEGF signaling to mimic the endogenous regulation. As shown in Fig. 3a, the addition of VEGF led to a rapid upregulation of p-ERK-1/2 (Thr202/Tyr204) and p-AKT (Ser473) in NC-shRNA transfected control cells. Strikingly, both of these effectors of VEGF signaling to mimic the endogenous regulation. As shown in Fig. 3a, the addition of VEGF led to a rapid (Fig. 2i), indicating a facilitating effect of Sec14l3 depletion on VEGFR2-Y1175 phosphorylation. Specifically, co-knockdown of PTP1B and Sec14l3 in HEK293T cells resulted in much higher phosphorylation levels of VEGFR2-cytosolic (Fig. 5c, d). To test whether this regulation is specific, cytoplasmic regions of three different VEGF receptors, VEGFR1-cytosolic, VEGFR2-cytosolic, and VEGFR3-cytosolic, were overexpressed with Sec14l3 for co-IP experiments respectively in HEK293T cells. Results showed that only VEGFR2-cytosolic could strongly interact with Sec14l3 (Fig. 5a), indicating that Sec14l3 somehow specifically regulates VEGFR2, but not VEGFR1 or VEGFR3 signaling. Taken together, these data indicate that zebrafish Sec14l3 is the counterpart of human Sec14l3 and both of them enhance VEGF signaling via regulating VEGFR2-Y1175 phosphorylation specifically.

Previous studies have demonstrated that decreased phosphorylation of VEGFR2-Y1175 may be caused by prolonged exposure of the activated VEGF2 to some tyrosine phosphatases beneath or on the plasma membrane. PTP1B has been reported and identified as such a specific phosphatase\(^ {21,22,41,42}\). To prove this notion, we compared the interaction between PTP1B and VEGFR2-cytosolic with or without Sec14l3 in HEK293T cells. The co-IP result showed that Sec14l3/Sec14l3 could indeed prevent VEGF2 from interacting with PTP1B (Fig. 5b). Then, we wondered whether knockdown of PTP1B could reverse the effect of Sec14l3 depletion on VEGFR2-Y1175 phosphorylation. Strikingly, co-knockdown of PTP1B and Sec14l3 in HEK293T cells resulted in much higher phosphorylation levels of VEGFR2-Y1175 compared to Sec14l3 knockdown alone (Fig. 5c, d).

Consistent with these in vitro results, overexpression of VEGF2-cytosolic mRNA or co-knockdown of ptp1b in sec14l3 morphants led to a relatively normal lumen size of DA and PCV at 25 and 30 hpf (Fig. 5e–h), while overexpression of VEGF2-cytosolic mRNA or knockdown of ptp1b alone had no obvious effect on the DA and PCV formation (Supplementary Figs. 8, 9). Taken together, these in vitro and in vivo observations imply that Sec14l3/Sec14l3 may somehow protect VEGFR2-Y1175 phosphorylation from its specific phosphatase PTP1B and then stimulate VEGF signaling.

VEGF2 trafficking is suppressed after Sec14l3 depletion. Based on the above results, in particular, the vesicle-like co-localization of Sec14l3 and VEGF2 in HUVECs (Fig. 1d), we hypothesize that Sec14l3/Sec14l2 may contribute to intracellular trafficking of VEGF2. To address this issue, we performed surface biotinylation assay to detect internalization and recycling of overexpressed VEGF2 in HEK293T cells (Supplementary Fig. 10). We found that, before induction of internalization, the biotin-labeled VEGF2 protein level on the cell surface in Sec14l3 shRNA transfected HEK293T cells was comparable...
to that in control cells (Fig. 6a, the first lane and fifth lane); following induction of internalization, intracellular biotin-labeled VEGFR2 proteins gradually increased in the control cells, but this increase was much slower in SEC14L2 shRNA cells (Fig. 6a). Thus, SEC14L2 is a potential regulator of VEGFR2 trafficking.

Next, the transport of VEGFR2-containing endosomes in HUVECs was examined by immunostaining assay. Before induction of internalization, endogenous VEGFR2 proteins on the cell surface were labeled similarly in control and SEC14L2 shRNA transfected cells (Fig. 6b, the first panel). After internalization induction at 37 °C for 10 min, the
Fig. 3 SEC14L2/sec14l3 knockdown attenuates VEGF signaling. a SECML2 knockdown counteracts VEGFa-motivated p-ERK and p-AKT levels in HUVECs. HUVECs were infected with NC or SECML2 shRNA for 48 h. After VEGFa stimulation for 5, 15 or 30 min, cell lysates were harvested and immunoblotted with indicated antibodies. b, c Statistical results of relative p-ERK (b) and p-AKT (c) levels in a. The grey intensity of each band was measured for calculating the ratio of p-ERK to ERK (b) and p-AKT to AKT (c). Data are then normalized to control group with 0 stimulation and represented as mean ± SEM from three independent experiments. d–e Depletion of sec14l3 decreases p-Erk (d) and p-Akt (e) levels in zebrafish embryos. Embryos from intercrossing sec14l3 heterozygous in Tg(kdrl:GFP)843Tg transgenic background were harvested at 23 hpf for p-Erk antibody immunostaining (d) or at 25 hpf for p-Akt antibody staining (e). DA, dorsal aorta. The ratio in the right corner indicates the number of embryos with reduced staining/the number of observed embryos. Scale bars, 50 μm. f The defective DA/PCV lumen formation in sec14l3 morphants can be partially rescued by ca-MEK or ca-AKT mRNA injection. Five nanograms std-MO or sec14l3-tMO2 in combination with 50 or 100 pg ca-MEK or ca-AKT mRNA was injected into one-cell stage embryos. Their morphology (the upper panel) or vasculature defects (the bottom panel) at 25 hpf are separately shown. The ratio in the right corner indicates the number of embryos with indicated morphology/the number of observed embryos. Scale bars, 50 μm. g Statistic results of the DA (upper panel) or PCV (bottom panel) luminal diameters of each group at 30 hpf. Twenty embryos of each group from one representative experiment were calculated and shown here. To quantify the DA and PCV luminal diameter for an embryo, five different vessel regions of the same fish were measured to calculate the mean value, which was used to represent its vessel diameter of this fish. Three independent experiments were carried out. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant. ANOVA tests were used for statistical analyses. Source data are provided as a Source Data file.

Fig. 4 SEC14L2 knockdown decreased VEGFR2 phosphorylation at Y1175 site. a Western blot analysis of cell lysates from NC or SEC14L2 shRNA infected HUVECs. Cells were infected and starved overnight, followed by 100 ng ml−1 VEGFa stimulation for 5, 15 or 30 min, respectively. VEGFR2 (p-Y1175), VEGFR2 (p-Y1054/1059), and total VEGFR2 levels were examined by immunoblotting using respective antibodies. b, c Statistical results of p-VEGFR2-Y1054/1059 (b) and p-VEGFR2-Y1175 (c) levels in a relative to total VEGFR2. Data are normalized to control group without stimulation and shown as mean ± SEM from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant. d, e Overexpression of zebrafish sec14l3 in HEK293T cells restores the level of p-VEGFR2-Y1175 inhibited by SEC14L2 knockdown. One or two micrograms sec14l3 plasmid was transfected into SEC14L2 knockdown stable HEK293T cells with VEGFR2 overexpression. Following starvation overnight, 100 ng ml−1 VEGFa was added and stimulation continues for 0, 5 or 15 min. Quantification data of the relative p-VEGFR2-Y1175 level from three repeated experiments are normalized to control group with 0 stimulation and shown on the right (e). *p < 0.05; **p < 0.01; ***p < 0.001; ns, not significant. The loading volume of a, d was adjusted for the same amount of total VEGFR2 level in each lane. Two-way ANOVA tests were used for statistical analyses in b, c, and e. Source data are provided as a Source Data file.
VEGFR2-containing endosomes in SEC14L2 knockdown HUVECs were significantly decreased compared to the control cells (Fig. 6b, c). Additionally, co-localization analysis between internalized VEGFR2 and the early endosomal marker EEA1 showed that SEC14L2 shRNA cells contained far fewer VEGFR2/EEA1 double positive endosomes than control cells (Fig. 6b, c), suggesting a requirement of SEC14L2 for VEGFR2 internalization into early endosomes.

It has been reported that in resting endothelial cells, VEGFR2 displays both the cell surface distribution and internal vesicular pool storage, which can be redistributed to the cell periphery upon VEGF stimulation. To investigate whether SEC14L2 participates in this process, we performed surface biotinylation assay over a 35-min time course (20 min internalization at 22 °C followed by 15 min recycling at 37 °C). In NC-shRNA control cells, about 40% of internalized VEGFR2 could be recycled back to the plasma membrane and this recycling rate was enhanced to approximately 80% in the presence of VEGFα stimulation (Fig. 6d), which is similar to the previous report. However, knocking down SEC14L2 drastically decreased the VEGFR2 recycling rate to about 8 and 20%, respectively, with or without VEGFα stimulation (Fig. 6d). Therefore, we conclude that SEC14L2 may promote VEGFR2 intracellular mobilization at least in two ways, internalization from and recycling back to the cell surface.
Furthermore, we tried to figure out the final destination of VEGFR2 after knocking down SEC14L2 in HUVECs. Western blotting results showed that the total VEGFR2 protein level is not significantly affected when SEC14L2 is depleted, exclusive of its possible role in VEGFR2 degradation (Supplementary Fig. 11). When co-staining VEGFR2 with late endosome markers, Rab7 or CD63, we found that SEC14L2 knockdown led to more VEGFR2 accumulation in these compartments (Fig. 6e, f). Taken these results together, we would like to prospect that more VEGFR2 is stored in late endosomes but not delivered to the lysosome for degradation in SEC14L2 deficient cells, which is consistent with previous reports12.

**SEC14L2/Sec14l3 physically interact with VEGFR2 and Rab5**

We next set out to investigate how Sec14l3/SEC14L2 mediate VEGFR2 trafficking. Given that Sec14l3 interacts with VEGFR2-cyto specifically (Fig. 5a) and SEC14L2 co-localizes with VEGFR2 endogenously in vesicular compartments (Fig. 1d), we first demonstrated that endogenous SEC14L2 indeed interacts with endogenous VEGFR2 and this interaction could be strengthened upon VEGFa stimulation (Fig. 7a), indicating that SEC14L2 prefers binding to activated VEGFR2. To further prove this point, wild-type form (WT-VEGFR2) or kinase mutation form (KM-VEGFR2) of VEGFR2 was co-transfected with Flag-tagged zebrafish Sec14l3 in HEK293T cells for Co-IP experiments43. We found that Sec14l3 associates with WT-VEGFR2 much stronger than KM-VEGFR2 (Fig. 7b, c). Taking these data together, we conclude that Sec14l3 favors associating with activated VEGFR2. Additionally, domain mapping results revealed that the Sec14 domain of Sec14l3 was essential for its interaction with VEGFR2 (Fig. 7d, e).

As a member of the receptor tyrosine kinases (RTKs) family, VEGFR2 is regulated by various Rab proteins at the intracellular trafficking level, which renders vesicle transport to particular destination somehow through recruiting their corresponding effectors44. Considering that Sec14l3/SEC14L2 act during VEGFR2 trafficking, we wondered whether Sec14l3 could associate with Rab family proteins. Four members of Rab proteins, representing distinct endocytosis routes45–47, were selected for co-IP experiments in HEK293T cells. Results revealed that only Rab4A and Rab5A could strongly interact with Sec14l3 (Fig. 7f), which coincides with the function of Sec14l3 in VEGFR2 internalization and recycling (Fig. 6). Furthermore, these interactions were confirmed by endogenous co-IP data in HUVECs (Fig. 7g, h). Domain mapping analyses disclosed that the N-terminal CRAL-TRIO domain and the C-terminal GOLD2 domain of Sec14l3 were required for interaction with Rab4A (Fig. 7d, i). Therefore, Sec14l3 may promote VEGFR2 endocytosis and recycling through its interaction with Rab5A and Rab4A.

**Sec14l3/SEC14L2 are required for Rab5A/4A activation**

Like other GTPases, the Rab5A/4A cycle between an active (GTP-bound) and an inactive (GDP-bound) state, which is of critical importance to modulate vesicle trafficking processes48,49. We wonder whether Sec14l3/SEC14L2 are required for the activity of Rab5A/4A to regulate VEGFR2 trafficking. To this end, we first compared the association of Sec14l3 with Rab5A/4A-GDP and Rab5A/4A-GTP. Transfection and in vitro binding assays revealed that Sec14l3 preferred binding to the GDP-bound state of Rab5A/4A (Fig. 8a, b). To further investigate the function of Sec14l3/SEC14L2 in Rab5A/4A regulation, we performed immunoprecipitation assay using a specific anti-Rab5A/4A-GTP antibody or pull-down assay using purified GST-R5BD, which specifically binds to Rab5A-GTP50. Compared to the NC-shRNA control, knocking down Sec14l3/SEC14L2 caused more than 50% reduction of the Rab5A-GTP and Rab4A-GTP amount (Fig. 8c, d), which is consistent with the compromised VEGFR2 internalization and recycling processes (Fig. 6). Taken together, these results suggest that Sec14l3/SEC14L2 facilitate the formation of Rab5A/4A in their GTP-bound active states, and then probably accelerate the movement of VEGFR2 vesicles to early endosomes and recycling endosomes to enhance VEGF signaling (Fig. 8e).

**Discussion**

Given the critical roles of endocytosis in VEGFR2 signaling, numerous relevant factors that have effects on receptor trafficking along endosomes have been identified, and most of them were demonstrated to participate in VEGFR2 internalization or degradation16,22,23,41,51–54. However, few factors were found to regulate a relatively complete VEGFR2 intracellular trafficking process, such as receptors internalization from and recycling back to the plasma membrane for enhancing signaling output. Here, we propose that the lipid binding proteins Sec14l3/SEC14L2 concurrently provoke VEGFR2 uptake from the plasma membrane and return from the endosomal pool to maximize signaling strength upon ligand stimulation. Mechanistically, Sec14l3/SEC14L2 conjugate activated VEGFR2 into Rab5-positive sorting endosomes and Rab4-positive fast recycling endosomes through their interaction to protect VEGFR2 from dephosphorylation by the peri-membrane tyrosine phosphatase PTP1B (Fig. 8e). Therefore, Sec14l3/SEC14L2 deficiency distorts VEGFR2 internalization and recycling, leading to a prolonged exposure to PTP1B and subsequent compromises of PLCγ/ERK and PI3K/AKT activation, which consequently results in defective...
vasculature morphogenesis in zebrafish embryos in vivo and vascular tubulogenesis in vitro. The effective induction of VEGF signaling through overexpression of cytoplasmic region of VEGFR2 mRNA or suppression of ptp1b expression or overexpression of constitutively active MEK/AKT partially restored the narrowed DA and PCV lumens in zebrafish sec14l3 morphants. This study provides insight into the regulation of VEGFR2 endocytosis in vertebrate vascular system formation.

It was apparent that sec14l3 inactivation specifically affected vasculogenesis, whereas the angiogenic process appeared to be nearly intact, although both processes heavily rely on VEGF signaling. The pattern of how sec14l3 is spatiotemporally...
expressed may provide an explanation for this. Before the onset of the major axial vessels at 19 hpf, sec14l3 is expressed in both arterial and venous progenitors. And thereafter, it is gradually restricted into the PCV compartment, not DA or ISVs. This particular time window is corresponding to vasculogenesis, but restricted into the PCV compartment, not DA or ISVs. This arterial and venous progenitors. And thereafter, it is gradually

- **PI3K/AKT and PLC/ERK signaling** has been well documented. Within the arterial-fated angioblast, VEGFR2 signaling induces PLC/ERK kinase cascade and subsequently stimulates expression of several genes in the Notch pathway to ensure the arterial cell fate. It has been reported that overexpression of constitutively active MEK or treatment with agonists of PLC/ERK signaling can rescue the DA formation in zebrafish embryos lacking vegfa. On the other hand, in the venous-fated angioblast, PI3K/AKT signaling is activated to antagonize ERK activity, and overexpression of constitutively active Akt promotes the venous cell fate. Therefore, it seems coincident with previous reports that overexpression of ca-MEK or ca-AKT could rescue the DA or PCV formation respectively in sec14l3 mutant embryos bearing a relatively low level of VEGF signaling. Since both PLC/ERK and PI3K/AKT pathways are downstream branches of the VEGF receptor, how they are preferentially activated in different endothelial progenitor cells or at different developmental stages remain intriguing questions. Furthermore, how Sec14l3 exactly regulates these processes also need to be investigated in the future.

Upon VEGF ligands stimulation, VEGFR2 is engaged in dimerization to induce phosphorylation of several tyrrosine residues in its cytoplasmic domain. Meanwhile, various phosphatases have been reported to remove tyrosine phosphorylation at distinct sites. Given that PTP1B specifically dephosphorylates VEGFR2 at the Y1175 site, we merely examined the rescue effect of PTP1B depletion following Sec14l3/SEC14L2 knockdown. However, we cannot exclude the possible involvements of other phosphatases, especially those transmembrane receptor-type PTPs, such as DEP1/CD148, VE-PTP and so on. Knocking down these phosphatases respectively in Sec14l3/SEC14L2 deficient embryos may provide more information.

Besides VEGF ligands, PECAM-1 mediated fluid shear stress signaling could also recruit VEGFR2 and then phosphorylate it at Y1175 site along with VE-Cadherin, thus regulating vascular remodeling, vascular homeostasis, cardiac development and atherogenesis. In this study, we didn’t focus on this branch, largely due to our in vivo observations that sec14l3 specifically exists and functions during zebrafish vasculogenesis before the onset of blood flow. Additionally, different from mammalian vascular development, it has been reported that changes in shear stress appear to be relatively unimportant in the initial stages of zebrafish angiogenesis because blood vessels can be successfully formed in the absence of heartbeat and blood flow until 14 days postfertilization. Even so, we still cannot exclude that Sec14l3 could participate in regulating fluid shear stress-induced VEGFR2 activation during later stages of zebrafish angiogenesis. It will be interesting to explore this possibility in the future.

Previous studies have shown that VEGFR2 endocytosis proceeds in a clathrin-dependent internalization, followed by the cascade of RAB recruitments to deliver VEGFR2 to the plasma membrane for recycling, into late endosome for storage, or into lysosome for degradation. In this work, we demonstrate that Sec14l3 could directly interact with RAB5A/4A and promote their GTP-bound states formation, and probably orchestrate VEGFR2 internalization and recycling back to the plasma membrane. After performing in vitro GEF assay based on mant-GTPyS fluorescence, we proposed that Sec14l3 could not act as a GEF to accelerate the GTP loading activity of RAB5 directly (Supplementary Fig. 12). Actually, whether Sec14l3 could regulate the GTP hydrolysis activity of RAB5A/4A also need to be determined by MESG-based single-turnover assay. In addition, whether the intrinsic GTPase activity of Sec14l3 is required for the activation of RAB5A/4A also awaits further investigation. Based on our data here, we would like to propose the following potential mechanisms of Sec14l2/Sec14l3 acting on RAB5A/4A activation.

1) Sec14l3 might serve as an adaptor to recruit a GEF protein for RAB5A/4A. Although we have not detected any interaction between Sec14l3 and several well-known GEF proteins of RAB5, such as Rabex5 and RABGEF1, we still cannot exclude this possibility, since there might be other unidentified GEFs for RAB5A/4A that could participate in this process.

2) Sec14l3 belongs to atypical class III PTPs, which are implicated in the traffic of phosphoinositides (PIs) between different membrane compartments. Different PI species display distinct subcellular distributions where they play intriguing functions. For example, PI3(3)P, PI(4,5)P2, and PI(3,5)P2 are found predominantly within early, recycling and late endosomes, respectively, which are essential for appropriate endosomal trafficking events. Drosophila PI 3-phosphatase MTMR13 promotes PI(3,5)P2 turnover at endosomes to activate Rab5 family member Rab21, PI(3,5)P2 levels in early endosomes may drive Rab5-RAB7 conversion and so on. Therefore, it deserves further exploration of whether Sec14l3 could also motivate specific phosphoinositides transport along vesicles to regulate endosome dynamics, either in morphology or in effector recruitment.
**Fig. 7** Sec14l3/SEC14L2 interact with VEGFR2 and RAB4A/5 via complementary domains. 

**a** SEC14L2 interacts with VEGFR2 endogenously in HUVECs. HUVEC cells were starved and stimulated with 100 ng ml\(^{-1}\) VEGFa for 5 min before harvest for IP and immunoblotting. TCL, total cell lysate. IgG serves as a negative control. Quantification of the interaction from three independent experiments is shown on the right (n = 3). *p < 0.05.

**b** Sec14l3 shows a much stronger association with WT-VEGFR2 than its kinase domain mutation form (KM-VEGFR2). Different forms of VEGFR2 were co-expressed with Flag-tagged Sec14l3 in HEK293T cells. WB, western blot. Quantification of the interaction is shown on the right (n = 3). t-tests were used for statistical analyses in a–c.

**d** Schematic diagrams of Sec14l3 and its truncated mutants. Numbers above the diagram indicate the corresponding amino acid positions. \(\Delta N\) indicates N-terminal CARL-TRIO domain deletion; \(\Delta S\), Sec14 domain deletion; \(\Delta G\), GOLD domain deletion.

**e** Sec14l3 interacts with VEGFR2 via its Sec14 domain. HEK293T cells were transfected with different Sec14l3 domain deletion forms with VEGFR2 plasmid respectively and harvested for IP.

**f** Sec14l3 specifically interacts with RAB4A and RAB5A. Different RAB plasmids were co-transfected with VEGFR2 into HEK293T cells respectively for co-IP assay.

**g** SEC14L2 interacts with RAB4a (g) and RAB5a (h) endogenously in HUVECs. i Sec14l3 interacts with RAB4A via its CRAL-TRIO and GOLD2 domains. Source data are provided as a Source Data file.
Methods

Zebrafish strains and embryos manipulation. Tuebingen strain of zebrafish (Danio rerio) was used. Unless otherwise stated, Tg(kdrl:GFP)s843Tg and Tg(fl1a:EGFP)y1 transgenic lines were used for easy observation of vasculature development. Ethical approval is acquired from Tsinghua University Animal Care and Use Committee for the fish maintenance and manipulations, according to the institutional animal care and use committee (IACUC) protocol (AP#13-MAM1). Embryos were raised and staged according to Kimmel et al.69. For injection, 1-cell stage embryos were harvested and injected with mRNAs or MOs in yolk for even distribution using the typical MPPI-2 quantitative injection equipment (Applied Scientific Instrumentation Co.). In this study, sec14l3, ca-AKT, and ca-MEK mRNA were synthesized using the mMESSAGE mMACHINE Kit (Ambion AM1344) and purified using the RNeasy Mini Kit (Qiagen). Morpholinos were ordered from Gene Tools, LLC. and prepared in stocks. sec14l3-tMO2, sec14l3-sMO, ptp1b-tMO, ptp1b-sMO and std-MO were synthesized by Gene Tools, LLC. The sequences of the used morpholinos are as follows: sec14l3-tMO2: 5′-ATGTCGCACGAGTGCAGCAGAAAT-3′; sec14l3-sMO: 5′-ATGTTTCTCACCTCTCAGCCATCTG-3′; ptp1b-tMO: 5′-CGATTTCCCGAAACTCGGCTTCCAT-3′;
pp1b-mCh: 5′-GAAGCCGAAACTGCTTATCTCCTGGT-3′ and std-MO: 5′-CTCTTACTCAGTCTTTACCTGGT-3′. Every single embryo was injected with 1 nl mRNA or MO solutions at indicated doses and raised to 19–30 hpf for observation. Embryos were harvested randomly for each experiment. For imaging of vasculature development, chorion was removed from embryos by Pronase K. Embryos were harvested randomly for each experiment. For living imaging of cell line was authenticated with STR profiling and culture. Its species origin was confirmed by PCR and culture. HUAECs cells (ScienCell #8010) were cultured in endothelial cell medium (ScienCell). Resource Center, Peking Union Medical College) were cultured in DMEM (Life Technologies). Primers for construction are listed in Supplementary Table 1. HEK293T cells (Cell Signaling Technology) or shRNA were cultured for 72 h and then washed twice by cold PBS, which was followed by incubation with the Alexafluor-47 conjugated anti-VEGFR2 antibody (#359909 Biolegend, 1:200) for 30 min at 4 °C. After removing extra antibody, cells were placed in an incubator at 37 °C for 30 min. After washing, un-reacted biotin was washed away by chilled PBS and labeled cells were immediately incubated at 37 °C to allow internalization for 5 min or 10 min. After internalization, culture medium was washed twice by ice-cold PBS, which was followed by incubation with 1% BSA twice, 1% BSA once and 20 mM iodoacetamide (IAA) once for 10 min to remove biotin-labeled proteins remaining on the plasma membrane. Then, cells were lysed for streptavidin beads pull-down assay. For VEGFR2 immunofluorescence assay, HUVECs infected with shRNA were cultured for 72 h and then washed twice by cold PBS, which was followed by incubation with the Alexafluor-47 conjugated anti-VEGFR2 antibody. After washing, cells were fixed with 4% paraformaldehyde for at least 12 h. After dehydration in methanol gradients, embryos could be stored at −80 °C. To measure the recycling of VEGFR2, transfected cells were washed and labeled at 4 °C with biotin for 30 min. The labeled cells were incubated with pre-warmed culture medium at 20 min at 22°C to allow cell surface proteins to internalize into RAB4-positive vesicles. The biotin-labeled proteins retained on the cell surface were removed by sequential washes with 10% FBS twice, 1% BSA once and 20 mM iodoacetamide (IAA) once, each for 10 min at 4 °C. Then cells were cultured at 37 °C for 30 min. At indicated time points, cells were washed with acid PBS (pH 2.5) for 3 times to remove un-internalized anti-VEGFR2 antibody on the cell surface. After washing, cells were fixed by 4% PFA and subjected to immunofluorescence. Antibodies used here include Sec14l2 (TA03723, ORIGENE, 1:100), EE1A (32828, Cell Signaling Technology, 1:100), VEGFR2 (#2479, Cell Signaling Technology, 1:200) and CD63 (3CBL553, Millipore, 1:100). For the measurement of VEGFR2, transfected cells were washed and labeled at 4 °C with biotin for 30 min. The labeled cells were incubated with pre-warmed culture medium for 20 min at 22°C to allow cell surface proteins to internalize into RAB4-positive vesicles. The biotin-labeled proteins retained on the cell surface were removed by sequential washes with 10% FBS twice, 1% BSA once and 20 mM iodoacetamide (IAA) once, each for 10 min at 4 °C. Then cells were incubated at 37 °C for 30 min. 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harvested for 2x loading buffer boiling and the samples were used for SDS-PAGE and Coomassie blue staining.

**Statistical analysis.** Results were analyzed with GraphPad Prism 5.0 and are presented as mean ± SEM/SD as indicated, and comparisons were performed between two groups using a two-tailed Student’s t-test or ANOVA test when comparing more than two conditions. For all analyses, *p < 0.05; **p < 0.01; ***p < 0.001 were considered statistically significant; ns indicated statistical non-significance with *p > 0.05. Each experiment was carried out at least three times independently. The sample size is pre-estimated to ensure statistical analysis and no sample was optionally excluded from analysis. No blinding was done in the analyses and quantifications.

**Data availability**

The authors declare that all data supporting the findings of this study are available within the article and its supplementary information files or from the corresponding author upon reasonable request. RNA-Seq raw data have been deposited in the GEO database and functional properties. **Free Radic. Biol. Med.** 34, 1458–1472 (2003).

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Acknowledgements
We thank Drs. Nathan D. Lawson (University of Massachusetts Medical School) and Bo Zhang (Peking University) for providing transgenic fishes and Wang Min (Yale University) for providing WT-VEGFR2 and KM-VEGFR2 plasmids. We also appreciate the helpful discussion and technical assistance from members of the Meng Laboratory. This work was financially supported by grants from the National Natural Science Foundation of China (#31522035, #91754112, #31801130 and #31371460), Major Science Programs of China (#2012CB945100).

Author contributions
B.G. performed most experiments, collected and organized data. Z.H.L. helped to culture cells for partial biochemical analysis. W.H.X., G.Y.L., and S.H.D. participated in a part of the work, including whole-mount in situ hybridization, constructs cloning and so on. A.M.M. and S.J.J. proposed and managed the project, and B.G., A.M.M., and S.J.J. designed the study and prepared the manuscript.

Additional information
Supplementary Information accompanies this paper at https://doi.org/10.1038/s41467-019-09604-0.

Competing interests: The authors declare no competing interests.

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Journal peer review information: Nature Communications thanks Shamshad Cockcroft and the other anonymous reviewer(s) for their contribution to the peer review of this work. Peer reviewer reports are available.

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