The RAS Guanyl Nucleotide-releasing Protein RasGRP1 Is Involved in Lymphatic Development in Zebrafish*[S]

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Background: RasGRP1 is a protein involved in Ras activation, but its physiological function is largely unknown.

Results: Knockdown of RasGRP1 impairs development of the lymphatic system in zebrafish embryos, shown as defective thoracic duct formation, pericardial and truck edema, and altered sprouts of lymphatic vessels.

Conclusion: RasGRP1 is involved in lymphangiogenesis in zebrafish.

Significance: RasGRP1 has a physiological function in early development.

The molecular basis of the lymphatic development remains largely unknown. Using zebrafish as a model, we discovered a novel role for the Ras guanine-releasing protein 1 (RasGRP1), a protein involved in Ras activation in lymphangiogenesis. Secondary lymphatic sprouts from the posterior cardinal vein give rise to thoracic duct which is the first lymphatic vessel in zebrafish. Knockdown of *rasgrp1* by injecting morpholino in zebrafish embryos impaired formation of thoracic duct accompanied by pericardial and truck edema, whereas blood vessel development of the embryos was largely unaffected. In *rasgrp1*-knockdown embryos, the number of sprouts producing the string of parachordal lymphangioblast cells was reduced. Meanwhile the total number of the secondary sprouts was not changed. As a result, the number of venous intersegmental vessels was increased, whereas the number of lymphatic vessel was reduced at a later stage. The lymphatic developmental defects caused by *rasgrp1* knockdown could be rescued by ectopic expression of a constitutively active HRas. Further analysis revealed that RasGRP1 knockdown could synergize with *flt4/vegfr3* knockdown to induce defects in lymphangiogenesis. Taken together, this finding demonstrates a critical role for RasGRP1 in lymphatic development in zebrafish.

The lymphatic vascular system regulates interstitial fluid homeostasis, fat absorption, and immune surveillance (1). Growing evidence has revealed that the lymphatic system is also involved in multiple disease processes such as lymphedema, tumor metastasis, inflammation, obesity, and hypertension (2). However, the molecular basis of lymphatic development remains poorly understood. During the last two decades, a few molecules and signaling pathways have been found to regulate differentiation of lymphatic endothelial cells and lymphatic migration, including the Sox18/Prox1 transcriptional pathway (3, 4), the VEGF-C/Flt4 signaling pathway (5, 6), Δ-like-4/Notch signaling (7), synectin (8), and collagen and calcium-binding EGF domain-1 (ccbe1) (9, 10). However, additional molecules contributing to lymphatic development remain to be discovered.

Ras guanine-releasing protein 1 (RasGRP1) is a member of the guanine nucleotide exchange factors that are involved in the activation of Ras proteins (11, 12). Previous studies on RasGRP1 were mainly focused on its regulation on Ras activation in lymphocytes (13). RasGRP1 is essential for T cell receptor signaling, thymocyte differentiation, and B cell proliferation (14–16). However, RasGRP1 likely possesses other physiological functions in addition to its regulation on lymphocytes. RasGRP1 is expressed in neurons in the brain (17) and in the kidney (18) in addition to its expression in a variety of blood cells, including T cells and B cells (14). However, whether or not RasGRP1 is involved in vascular or lymphatic development remains elusive. Our previous work revealed that PAQR10/11, a member of the progestin and AdipoQ receptor (PAQR)[2] family, imparts a spatial regulation on the Ras signaling pathway and that RasGRP1 is implicated in the regulation (19). We next analyzed the potential physiological function using the zebrafish system and discovered that PAQR11 is involved in early heart development in zebrafish embryos (20). In this study, we analyzed the potential function of RasGRP1 during zebrafish development and discovered a novel function of RasGRP1 in early lymphangiogenesis.

EXPERIMENTAL PROCEDURES

Maintenance of Zebrafish—WT AB and *Tg (fli1:EGFP)^y1* zebrafish (21) (obtained from J. Du, Institute of Neuroscience, Chinese Academy of Sciences, Shanghai, China) were maintained under standard conditions. Zebrafish studies were

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[S] This article contains supplemental Figs. 1–12.

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[2] The abbreviations used are: PAQR, progestin and AdipoQ receptor; MO, morpholino; dpf, days post-fertilization; hpf, hours post-fertilization; DA, dorsal aorta; PCV, posterior cardinal vein; Pl, cardinal vein plexus; TD, thoracic duct; eGFP, enhanced GFP; PL, parachordal lymphangioblast; ISV, intersegmental vessel; vISV, venous intersomitic vessel.
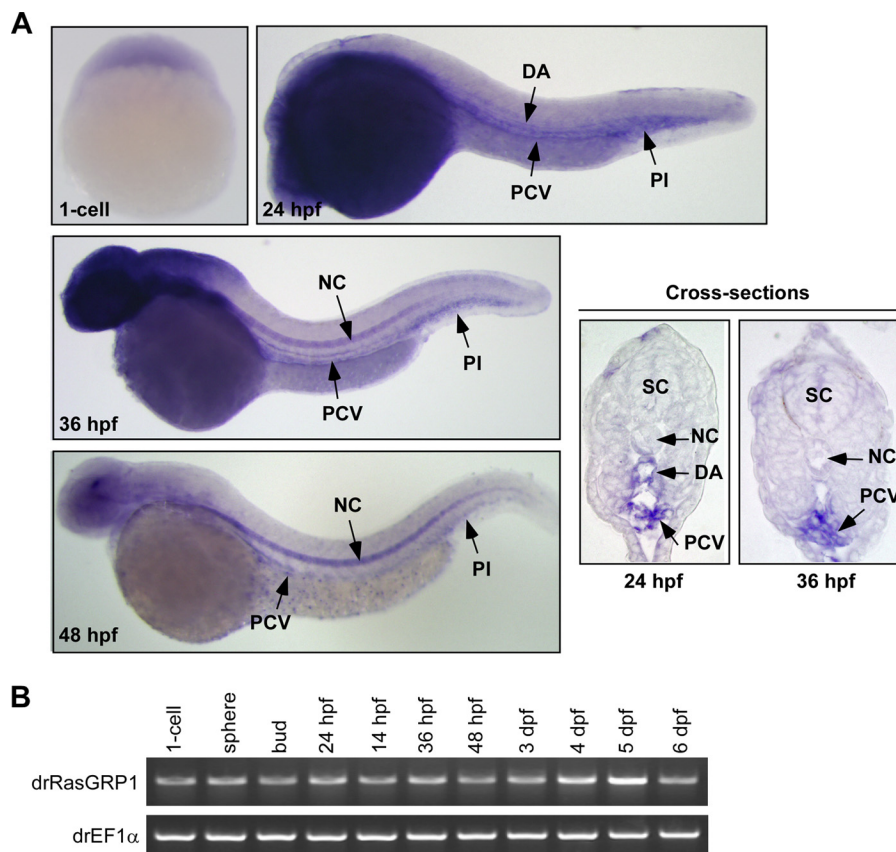
FIGURE 1. **RasGRP1 is expressed in PCV in zebrafish.** *A*, WISH was used to analyze the embryonic expression pattern of RasGRP1. At 24 hpf, RasGRP1 expression was detected in the DA, PCV, and PI region of the embryos. At 36 and 48 hpf, RasGRP1 expression was restricted to the PCV and PI region. From 36 hpf on, RasGRP1 was also detected in the notochord (*NC*) and head region. A cross-section of stained embryos showed DA and PCV expression of RasGRP1 at 24 hpf and PCV expression at 36 hpf. *SC*, spinal cord. *B*, the expression of RasGRP1 was analyzed by RT-PCR from the 1-cell stage to 6 dpf. The *rasgrp1* mRNA (a 1059-bp PCR product) is shown in the *top gel*, and the mRNA of EF1α (a 524-bp PCR product) served as an internal control (*bottom gel*).

approved by the Institute of Neuroscience, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, Shanghai, China.

Morpholino and Microinjection—Morpholinos (MOs) were purchased from Gene Tools (Philomath, OR). The first *rasgrp1* morpholino (RasGRP1-MO) was designed to cover the start codon region and 5′ untranslated region with a sequence of 5′-TGCGATTCATGGTGTCAAATCCCAT-3′. The second *rasgrp1* morpholino (RasGRP1-MO2) was targeted at a splice junction between exon 13 and intron 13 of *rasgrp1* pre-mRNA with a sequence of 5′-GTTGGTTTAAATACGTACAAATC-CT-3′. The *flt4* morpholino (Flt4-MO) was used as reported previously with a sequence of 5′-CTCTTCATTTCCAG-GTTTCAAGTCC-3′ (8). The control morpholino was a standard control MO with no target in zebrafish embryos, and its sequence was 5′-CCTCTTACCTCAGTTACAATTTATA-3′. The morpholinos were diluted in nuclease-free water (Ambion, Austin, TX), pressure-injected into 1- or 2-cell-stage zebrafish embryos using an mn-151 joystick micromanipulator and an IM-300 microinjector (Narishige, Japan). RasGRP1-MO was injected at a concentration of 6 ng/embryo, and RasGRP1-MO2 was used in 14 ng/embryo after titration with various concentrations. Flt4-MO was injected at a concentration of 1 ng/embryo. A lymphatic dye uptake assay was performed as described previously (22). In brief, embryos at 6 days post-fertilization (dpf) were injected below the skin in the trunk region with a

fluorescent dye Alexa-Fluor 568 dextran (Invitrogen) using glass capillary needles and were photographed 3–6 h after injection.

Probe Synthesis and Whole-mount in Situ Hybridization—Probes labeled with digoxigenin (Roche) were synthesized with linearized plasmids by using Sp6 or T7 RNA polymerase (Ambion). Whole mount *in situ* hybridization (WISH) was carried out as described previously (23). For cross-section analysis, the stained embryos were treated with 75, 80, 95, and 100% ethanol, followed by paraffin treatment before being embedded in paraffin and sectioned (8 μm in thickness) using a Lecia RM 2126 microtome.

Preparation and Injection of Zebrafish rasgrp1 mRNA—To make a morpholino-resistant form of RasGRP1, a full-length zebrafish RasGRP1 expression plasmid was generated to contain five same-sense mutations localized in the targeting region of RasGRP1-MO. The *rasgrp1* mRNA was generated with the mMESSAGE mMACHINE kit (Ambion). In the rescue experiments, each 1-cell stage embryo was injected with 1–5 pg of synthesized zebrafish *rasgrp1* mRNA.

Microscopy and Imaging—The zebrafish embryos were mounted in 1.0–1.5% low-melt agarose for observation and photographing. The images were acquired by a laser-scanning confocal microscope (Zeiss LSM 510, Zeiss, Jena, Germany) or Olympus DP72 (Tokyo, Japan).
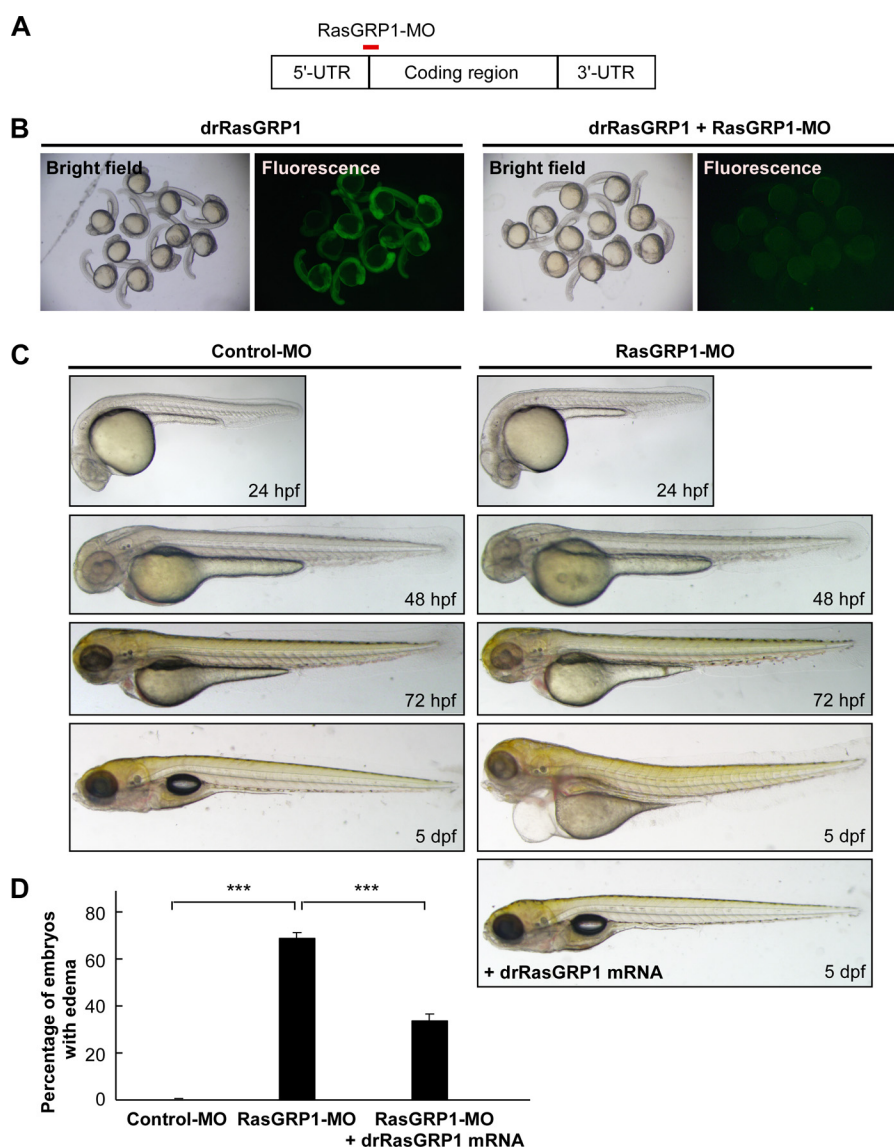
A

RasGRP1-MO

| 5'-UTR | Coding region | 3'-UTR |

B

drRasGRP1	drRasGRP1 + RasGRP1-MO
Bright field — Fluorescence	Bright field — Fluorescence

C

Control-MO	RasGRP1-MO
24 hpf	24 hpf
48 hpf	48 hpf
72 hpf	72 hpf
5 dpf	5 dpf
	+ drRasGRP1 mRNA 5 dpf

D

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Percentage of embryos with edema

Control-MO   RasGRP1-MO   RasGRP1-MO + drRasGRP1 mRNA

FIGURE 2. **Knockdown of *rasgrp1* results in severe edema.** *A*, RasGRP1-MO was designed to block the translation of RasGRP1 by targeting at the start codon region (+1 to +25). *B*, embryos injected with drRasGRP1-GFP mRNA (50 pg) had expression of green fluorescent signals. Coinjection of RasGRP1-MO (6 ng) with drRasGRP1-GFP mRNA (50 pg) abolished drRasGRP1-GFP expression. The data were obtained from 28-hpf embryos. *C*, zebrafish embryos were injected with control MO (6 ng) or RasGRP1-MO (6 ng), and the embryonic development was monitored at 24, 48, 72, and 5 dpf. Note that severe edema occurred in the pericardial sac and trunk region at 5 dpf in the RasGRP1 morphants and that such defects were rescued by coinjection of a morpholino-resistant drRasGRP1 mRNA (1 pg). *D*, the percentage of embryos with edema was calculated from control MO-injected embryos ($n = 241$), RasGRP1-MO morphants ($n = 288$), and the embryos coinjected with RasGRP1-MO and drRasGRP1 mRNA ($n = 207$). The data are shown as mean $\pm$ S.D. ***, $p < 0.001$ by Student's *t* test.

Statistic Analysis—The phenotypic severities of the zebrafish morphants was analyzed by Chi square analysis. The statistic difference between two groups was analyzed by unpaired Student's *t* test.

RESULTS

Expression Profile of rasgrp1 during Zebrafish Development—We used WISH and RT-PCR in wild-type zebrafish embryos to investigate the spatial and temporal expression pattern of *rasgrp1*. Both WISH and RT-PCR results revealed that *rasgrp1* was maternally provided, as its mRNAs were detected at the 1-cell stage before onset of zygotic gene expression (Fig. 1). From 24 h post fertilization (hpf) on, restricted expression of *rasgrp1* was observed in the dorsal aorta (DA), posterior cardinal vein (PCV), and cardinal vein plexus (Pl) regions. The signal of *ras-*

grp1 expression was mostly lost in DA but still persistent in PCV and Pl regions at 36 hpf (Fig. 1*A*), at a time when the lymphatic precursors begin to migrate toward the horizontal myoseptum region from the PCV (9). By 48 hpf, *rasgrp1* expression in the PCV and Pl regions was decreased markedly (Fig. 1*A*). In addition, we found apparent staining of *rasgrp1* in the notochord from approximately at 36 hpf, as well as in the head region (Fig. 1*A*). In control experiments, no hybridization signal was seen in the DA, PCV, and Pl regions with rasgrp1 sense probe (supplemental Fig. 1). The tissue-specific expression pattern of RasGRP1 in endothelia cells and the nervous system is consistent with the findings in mammals (17). Besides, we analyzed *rasgrp1* mRNA expression in zebrafish embryos at different stages from the 1-cell stage to 6 dpf using RT-PCR (Fig. 1*B*). It appeared that *rasgrp1* was expressed in all stages of early
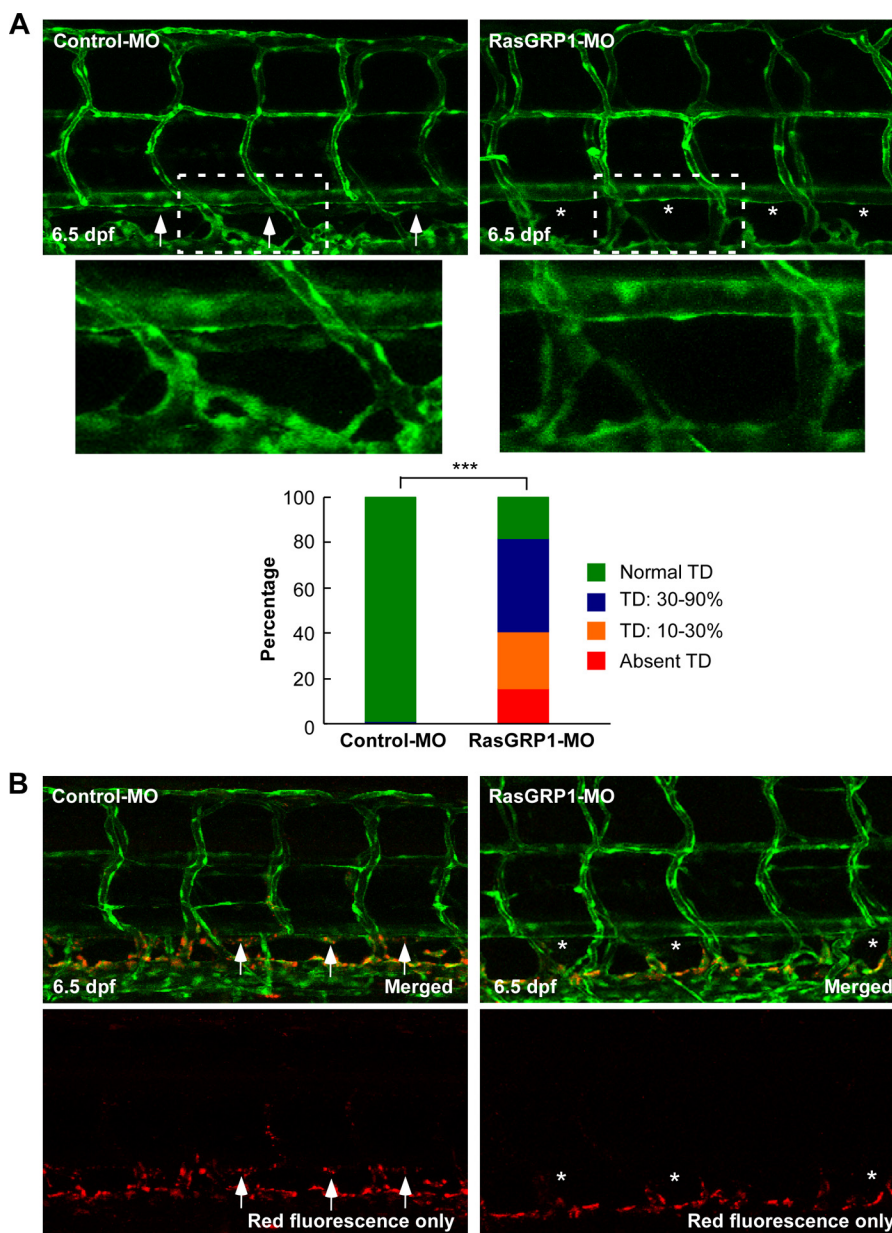
FIGURE 3. **Knockdown of *rasgrp1* leads to defects in thoracic duct formation.** *A*, representative confocal images of 6.5 dpf in *Fli1:eGFP^y* zebrafish embryos injected with control MO or RasGRP1-MO. Normal TD formation in the control embryos is marked by *arrows*, and the absence of the TD in RasGRP1-MO-injected embryos is marked by *asterisks*. The *insets* are magnified pictures of the *dotted-line boxes*. TD formation was quantitatively analyzed by measuring its presence from segments of somite 5 to somite 15, and the result is shown in the *bottom panel* ($n = 120$ for control embryos, and $n = 187$ for RasGRP1 morphants). ***, $p < 0.001$ by Chi square analysis. *B*, lymphatic dye uptake assay in *Fli1:eGFP^y* embryos. Alexa Fluor 568 dextran (*red*) was injected into zebrafish embryos at 6 dpf, and the uptake of the dye was found in control embryos (*arrows*) but not in RasGRP1-MO-injected embryos (*asterisks*).

embryos and larvae up to 6 dpf (Fig. 1*B*). These data, therefore, indicate that RasGRP1 may play a functional role in the development of neural system, notochord, vascular, or lymphatic vessels.

Knockdown of rasgrp1 Results in Pericardial and Truck Edema—To study the roles of RasGRP1 during early zebrafish embryogenesis, we used an antisense morpholino oligonucleotide (RasGRP1-MO) to block the translation of RasGRP1 (Fig. 2*A*). To validate the knockdown efficiency, we constructed a fusion gene, drRasGRP1-GFP, with the 5′ untranslated region sequence of *rasgrp1* that included the targeting sequence of RasGRP1-MO and a GFP reporter. The expression of the GFP fusion protein was abolished when RasGRP1-MO was injected

into the zebrafish embryos, indicating efficient silence of *rasgrp1* expression by the morpholino (Fig. 2*B*). As *rasgrp1* was expressed in the vascular/lymphatic system during early zebrafish development (Fig. 1), we investigated the possible function of RasGRP1 in the development of vessel systems. The zebrafish embryos injected with 10 ng or a higher dose of RasGRP1-MO exhibited blood accumulation, curl tails, and blood flow retardation (supplemental Fig. 2). These defects could be partially rescued by *rasgrp1* mRNA (supplemental Fig. 2) that was mutated in the region targeted by RasGRP1-MO so that it was resistant to the morpholino. Intriguingly, embryos injected with a lower dose of RasGRP1-MO (at 6 ng/embryo) gradually developed severe edema in the pericardial sac and around gut

after 4 dpf (Fig. 2*A*), resembling the phenotype of thoracic duct (TD) defect, as described previously (9, 22). However, these morphants showed normal morphology during the first 3 days of development (Fig. 2*C*). In summary, about 34% of RasGRP1 morphants developed edema at 5 dpf (Fig. 2*D*). In contrast, almost all embryos injected with 6 ng of control morpholino developed normally (Fig. 2*D*). To assure the specificity of Ras-GRP1-MO in the induction of edema, we analyzed another morpholino that affected splicing of *rasgrp1* mRNA (RasGRP1-MO2, supplemental Fig. 3). Injection of the zebrafish embryos with RasGRP1-MO2 was able to successfully disrupt the splicing of *rasgrp1* mRNA (supplemental Fig. 2). As expected, similar edema phenotypes were observed with RasGRP1-MO2 (supplemental Fig. 4). Furthermore, coinjection of *rasgrp1* mRNA (at 1 pg/embryo) could significantly rescue the edema phenotype caused by RasGRP1-MO (Fig. 2*B*). Taken together, these observations indicate that RasGRP1 likely plays an important role in zebrafish lymphatic development.

Rasgrp1 Knockdown Leads to Defects in Thoracic Duct Formation—To explore whether RasGRP1 played a functional role in lymphatic development, we silenced RasGRP1 in *Fli1:eGFP^{y1}* zebrafish embryos in which enhanced GFP (eGFP) was expressed in both blood and lymphatic vessels (21). We first examined the formation of the TD, which was the first and largest lymphatic vessel formed between the DA and PCV. The TD was analyzed by measuring its length in 10 somites (supplemental Fig. 5) as described previously (7, 8). Upon injection of 6 ng of RasGRP1-MO, 16% of the embryos failed to form any TD by 6.5 dpf, a stage when this vessel is fully formed (22). In 25% of RasGRP1-MO-injected embryos, only 10% to 30% of TD was formed, whereas 40% of the morphants had 30% to 90% TD formation (Fig. 3*A*). In contrast, TD was completely formed in 93% of control morpholino-injected embryos by 6.5 dpf (Fig. 3*A*). Similar results were also observed in RasGRP1-MO2-injected embryos (supplemental Fig. 6).

To confirm the lack of TD formation in RasGRP1-MO-injected embryos, we performed a subcutaneous dye uptake experiment by injecting zebrafish embryos at 6.5 dpf blow the skin in the trunk region with a fluorescent dye Alexa Fluor 568 dextran. The Alexa Fluor 568 dextran was absorbed by the TD in control embryos but not in the RasGRP1-MO-injected embryos (Fig. 3*B*). These results, therefore, provide further evidence that TD formation is disrupted by *rasgrp1* knockdown in the zebrafish embryos.

Knockdown of rasgrp1 in Zebrafish Does Not Affect Angiogenesis—To verify that the observed lymphatic defects caused by RasGRP1 knockdown was not because of a secondary effect of angiogenic defects, we investigated whether knockdown of RasGRP1 could impair early vascular development. We did not observe any apparent cardiovascular defects upon injection of 6 ng of RasGRP1-MO (Fig. 4*A*). Furthermore, arteriovenous differentiation of the large axial vessels was apparently not altered in the RasGRP1-MO-injected embryos, as evidenced by normal expression patterns of arterial and venous markers including Flt4, Ephrin B2, and Eph B4 at 28 hpf (Fig. 4*B*). Similarly, no vascular defects were observed with Ras-GRP1-MO2-injected embryos (supplemental Fig. 7). Collectively, these data indicate that the lymphatic defects upon *ras-*
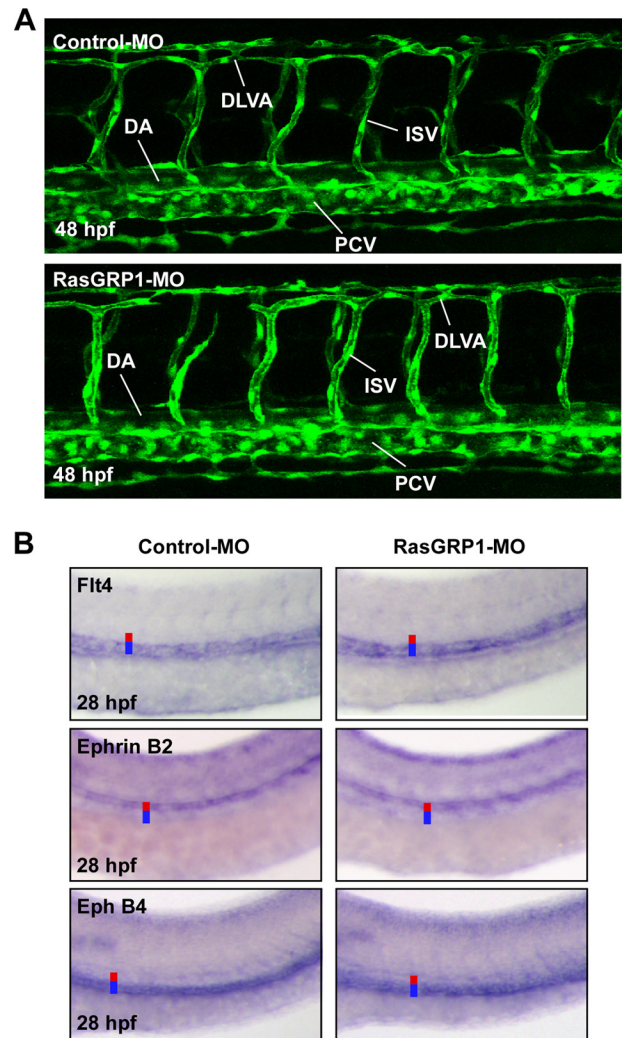
FIGURE 4. **Knockdown of *rasgrp1* does not affect angiogenesis.** *A*, representative confocal images of 48 hpf *Fli1:eGFP^y* zebrafish embryos. *DLAV*, dorsal longitudinal anastomosing vessel. *B*, WISH was performed with control MO- and RasGRP1-MO-injected embryos 28 hpf to analyze expression of the arterial marker (*Ephrin B2*) and venous markers (*Flt4, Eph B4*). *Red lines* denote the DA, and *blue lines* denote the PCV.

grp1 knockdown are not due to a secondary effect of disrupted angiogenesis by the morpholino.

Knockdown of rasgrp1 Impairs Early Lymphatic Development—We next analyzed whether knockdown of RasGRP1 could impair development of the parachordal lymphangioblast (PL) cells, which contribute to TD formation as they are precursors for lymphatic vessels (9). Similar to TD analysis, PL string formation was analyzed by measuring its length in 10 somites. At 60 hpf in control embryos, PL cells were detected in nearly every somite segment (Fig. 5*A*). In contrast, PL cells were absent in 44% of RasGRP1-MO-injected embryos, whereas 22% of embryos had 10–30% of the normal length of PL cells, and 21% embryos had 30–90% of the normal length of PL cells (Fig. 5*A*). Similarly, the embryos injected with RasGRP1-MO2 were also defective in PL cell development (supplemental Fig. 8). These data, therefore, provide additional evidence that Ras-GRP1 is involved in early lymphatic development in zebrafish embryos.
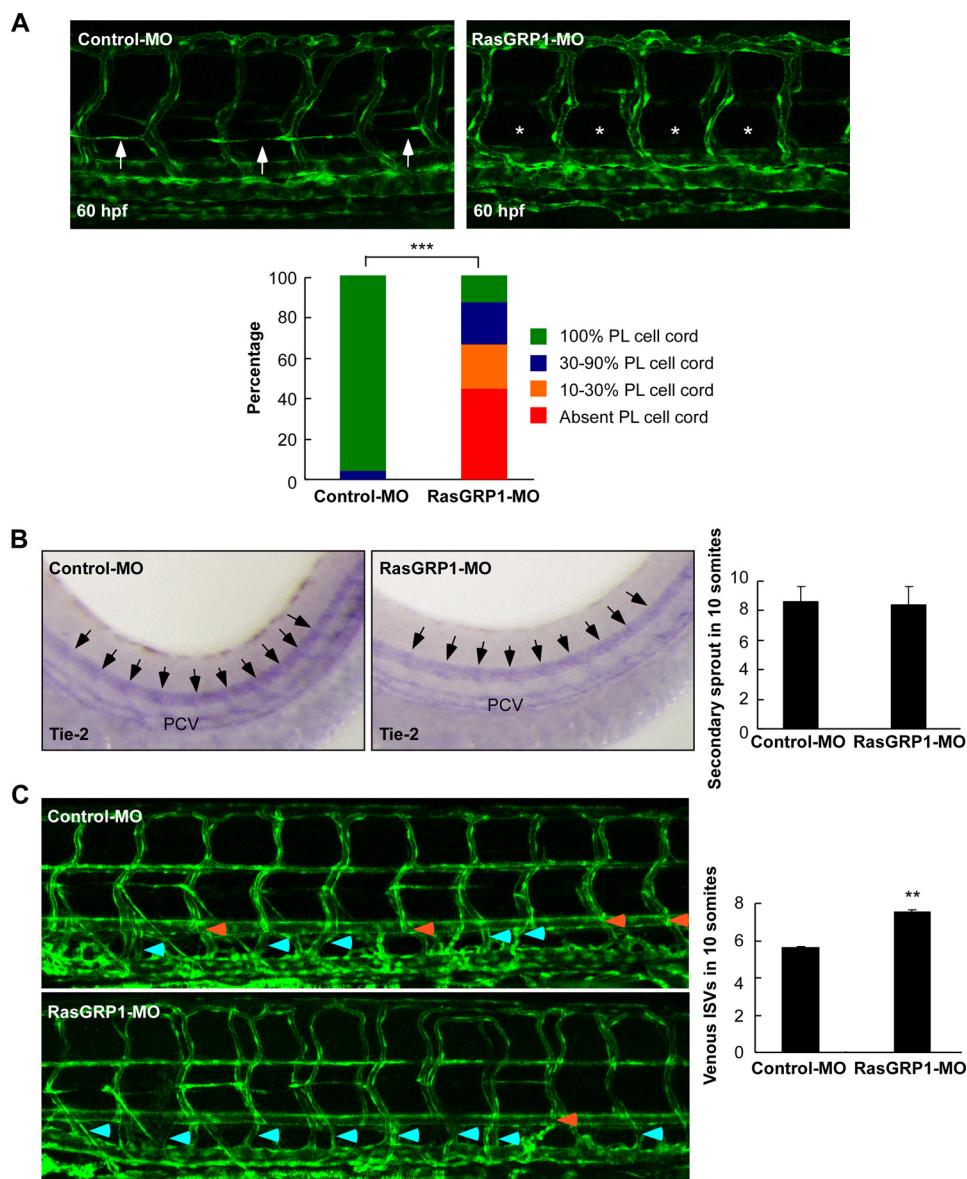
FIGURE 5. **Knockdown of *rasgrp1* impairs early lymphatic development in zebrafish.** *A*, representative confocal images of *Fli1:eGFP*[y] zebrafish embryos at 60 hpf upon injection of control MO or RasGRP1-MO. The presence of a PL string in control embryos is marked by *arrows*, and its absence in RasGRP1 morphants is marked by *asterisks*. The percentage of PL cell formation was quantitatively analyzed by measuring its presence in 10 somites, and the results are shown in the *lower panel* ($n = 121$ for control and $n = 245$ for RasGRP1 morphants). ***, $p < 0.001$ by Chi square analysis. *B*, WISH of control embryos and RasGRP1 morphants at 48 hpf for *Tie-2* expression. The secondary sprouts are marked by *arrows*. The number of secondary sprouts in 10 somites at 48 hpf was quantified ($n = 10$ for control embryos and $n = 10$ for RasGRP1-MO-injected embryos). The data are shown as mean $\pm$ S.D. There is no significant difference between the two groups. *C*, quantification of unilateral venous ISVs that connect to PCV in *Fli1:eGFP*[y] zebrafish embryos at 6 dpf. The number of venous ISVs was measured from the 10-somite region of control embryos ($n = 71$) and RasGRP1-MO-injected embryos ($n = 107$). The data are shown as mean $\pm$ S.D. **, $p < 0.01$ by Student's *t* test. Representative confocal images of *Fli1:eGFP*[y] zebrafish embryos at 6 dpf are shown in the *left panels*. *Light blue arrows* indicate venous ISVs, and *orange arrows* indicate arterial ISVs.

To determine whether the absence of PL cells was caused by lymphangiogenic sprouts defects, we analyzed the expression of *Tie2*, which marks both angiogenic and lymphangiogenic secondary sprouts at 48 hpf. The WISH results revealed that RasGRP1-MO-injected embryos had normal the total number of secondary sprouts compared with control embryos (Fig. 5*B*). We also counted the numbers of intersegmental vessels (ISVs) that connected to the PCV in *Fli1:eGFP*[y1] embryos at 6 dpf. It appeared that the proportion of venous intersomitic vessels (vISVs) in *rasgrp1* morphants was increased significantly (Fig. 5*B*). In control embryos, about 50% of the ISVs were vISVs, whereas about 75% of ISVs were vISVs in *rasgrp1* morphant

embryos (Fig. 5*C*). Because vISVs are formed through connection of secondary angiogenic sprouts, the rest of the sprouts should be the secondary lymphangiogenic sprouts contributing to TD formation. An increase in the number of vISVs would indicate a decrease in secondary lymphangiogenic sprouts forming the TD, in agreement with the finding that TD formation was disrupted by *rasgrp1* knockdown (Fig. 3). Taken together, these results demonstrate that RasGRP1 is required for early lymphangiogenesis in zebrafish.

Activated HRas Rescues Lymphatic Defects Caused by Ras-GRP1 Knockdown—Next we tried to explore the molecular basis underlying RasGRP1 knockdown-mediated lymphatic
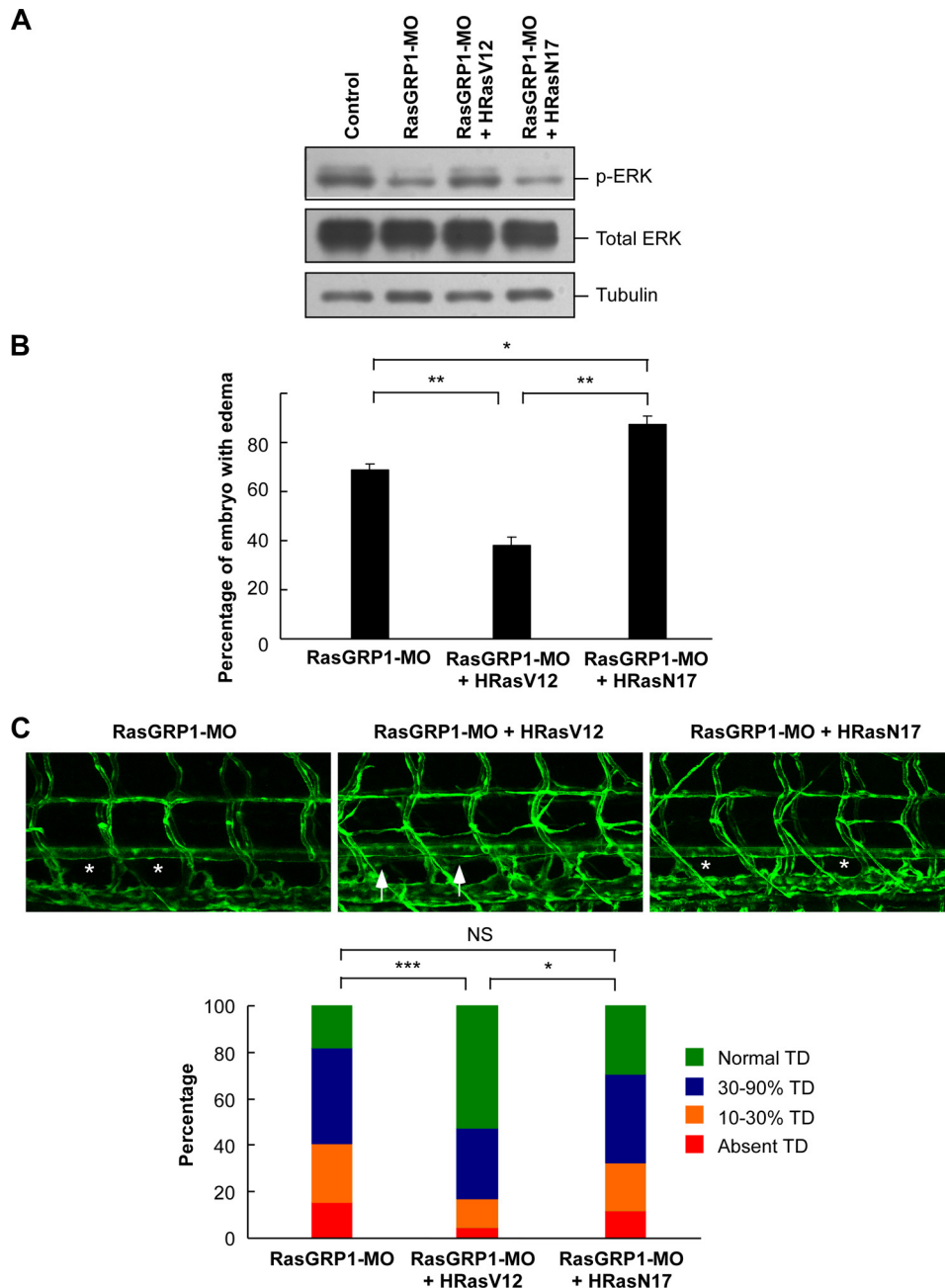
FIGURE 6. **The lymphatic defects upon *rasgrp1* knockdown is rescued by activated Hras.** *A*, Western blot analysis for ERK phosphorylation. Zebrafish embryos were injected with the mRNAs as indicated, and the whole embryo protein extract was used in Western blotting to detect the levels of phosphorylated ERK1/2 and total ERK1/2. The doses of injection were as follows: 6 ng/embryo for control-MO and RasGRP1-MO, 1 pg/embryo for HRasV12 mRNA, and 2.5 pg/embryo for HRasN17 mRNA. *B*, the percentage of edema at 5 dpf. The data are shown as mean ± S.D. **, indicates $p < 0.01$ by Student's *t* test. *C*, representative confocal images of 6.5 dpf *Fli1:eGFP[y]* zebrafish embryos. The properly formed TD is marked by *arrows*, and the absence of TD is marked by *asterisks*. *D*, quantification of TD formation at 6.5 dpf embryos ($n = 187$ for RasGRP1-MO, $n = 165$ for RasGRP1-MO plus HRasV12, and $n = 195$ for RasGRP1-MO plus HRasN17). *, $p < 0.05$; ***, $p < 0.001$; *NS*, not significant; Chi square test.

vessel defects during early development in zebrafish. As RasGRP1 is an activator of Ras proteins, we thus analyzed whether RasGRP1 affects lymphangiogenesis through modulation of Ras/ERK signaling. As expected, ERK phosphorylation was reduced by RasGRP1-MO in zebrafish embryos at 36 hpf (Fig. 6A). We constructed a constitutively active HRas (HRasV12) and a dominant negative HRas (HRasN17) (24). When these two Ras constructs were expressed in zebrafish embryos, they could alter ERK phosphorylation, consistent with the activity of the Ras proteins (Fig. 6A and supplemental Fig. 9). In agreement

with these observations, we also found that ERK phosphorylation in the PCV region was reduced by RasGRP1-MO (supplemental Fig. 10). As *rasgrp1* knockdown is associated with a decrease in Ras/ERK signaling, we hypothesized that the lymphatic developmental defects caused by *rasgrp1* down-regulation would be rescued by the constitutively active Ras. As expected, the percentage of edema caused by RasGRP1-MO was reduced significantly in the embryos coinjected with HRasV12 mRNA, whereas the percentage of edema was increased in embryos coinjected with HRasN17 mRNA (Fig.
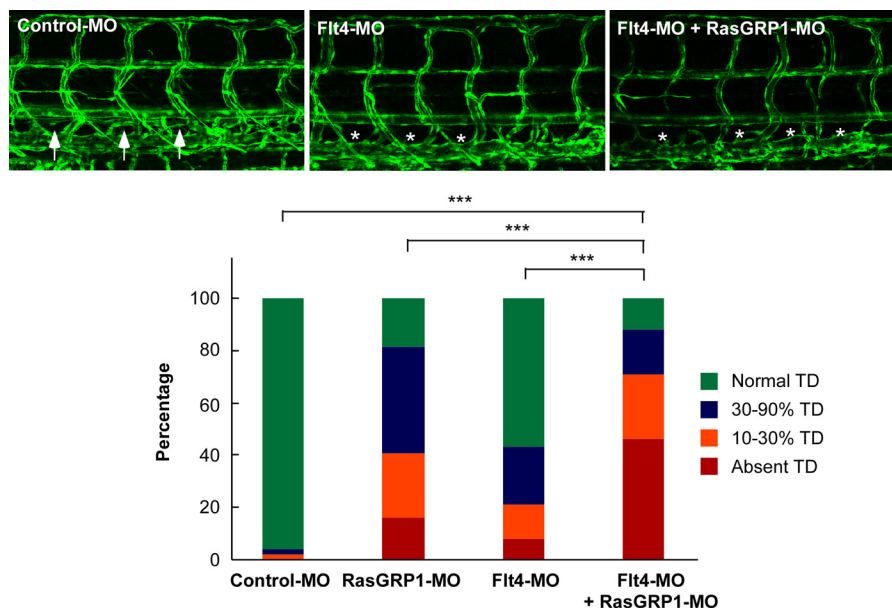
FIGURE 7. **RasGRP1 genetically interacts with Flt4.** Representative confocal images of 6.5 dpf *Fli1:eGFP^y* zebrafish embryos injected with control MO, Flt4-MO (1 ng), and RasGRP1-MO (6 ng) as indicated. The normally formed TD is marked by *arrows*, and the absence of TD is marked by *asterisks*. TD formation was quantitatively analyzed ($n = 52$ for control embryos, $n = 187$ for RasGRP1-MO, $n = 121$ for Flt4-MO, and $n = 138$ for RasGRP1-MO plus Flt4-MO). ***, $p < 0.001$ by Chi square analysis.

6*B*). We also analyzed the effect of coexpression of Ras proteins on TD formation in *Fli1:eGFP^{y1}* zebrafish at 6.5 dpf. Consistently, HRasV12 could partially rescue the TD defects induced by RasGRP1-MO, whereas HRasN17 could not rescue the defects (Fig. 6*C*). The percentage of embryos without any TD formation upon *rasgrp1* knockdown was reduced from 15.9% to 4.5% by HRasV12 overexpression (Fig. 6*C*). Collectively, these data revealed that an alteration of Ras/ERK signaling pathway is, at least partially, implicated in the observed lymphatic developmental defects caused by *rasgrp1* knockdown.

Synergistic Effect of Silencing Both Flt4/VEGFR3 and Ras-GRP1 on Lymphatic Development—VEGFC and Flt4 (VEGFR3) are known to be essential for the formation of TD in zebrafish (25). Recently, it has been demonstrated that Ras proteins regulate lymphatic vessel growth by modulating Flt4 signaling in mice (26). As RasGRP1 is an activator of Ras proteins, we then explored whether RasGRP1 genetically interacts with Flt4. We used a morpholino oligonucleotide to block the translation of *flt4*, as characterized previously (8). We found that a low dose of Flt4-MO induced a minimal defect in TD formation, consistent with previous findings (8). Notably, knockdown of both *flt4* and *rasgrp1* caused much more severe defects in lymphatic development than knockdown of either gene alone (Fig. 7), shown as absence of TDs in 46% of embryos with double knockdown (Fig. 7). Thus, our findings provide a preliminary model that Ras-GRP1 may have a genetic interaction with Flt4 during lymphatic development in zebrafish.

DISCUSSION

So far there are four RasGRP genes identified in mammals, whereas zebrafish have three RasGRPs, including RasGRP1, RasGRP2, and RasGRP3 (27). The physiological functions of the RasGRP family are largely unknown. RasGRP1 has been found to be required for T cell receptor signaling, thymocyte

differentiation, and B cell proliferation (14–16). RasGRP2 is required for proper platelet aggregation in mice (28), whereas RasGRP3 was reported to regulate angiogenesis in response to VEGF stimulation (29). However, mice depleted of *rasgrp1* were phenotypically normal except for defects in T cell development (15, 16). This study reveals for the first time a new physiological function of RasGRP1 in lymphatic development in zebrafish embryos. Knockdown of *rasgrp1* impaired the formation of lymphangiogenic sprouts, development of PL cells, and formation of TD. It is worth noting that at a low dose of RasGRP1-MO, the development of blood vessels was not affected (Fig. 4), and the development of notochord, somite, and heart are also not affected (supplemental Fig. 11). Meanwhile, injection of a high dose of rasgrp1 mRNA appeared to have no effect to promote lymphangiogenesis in zebrafish embryos (supplemental Fig. 12). Interestingly, silencing of both rasgrp1 and flt4/vegfr3 in zebrafish embryos aggravated the lymphatic defects. Collectively, these observations have pinpointed a critical role of RasGRP1 in lymphatic development in zebrafish.

Zebrafish is a well established model to study lymphatic development. According to previous reports (8, 9, 22, 30), TD development is initiated at ~36 hpf when the secondary sprouts branch off from the PCV. About half of these sprouts are angiogenic, and they further develop into venous ISVs, whereas the other half of the sprouts are lymphangiogenic and further develop into the string of PC cells at the horizontal myoseptum region. Beyond 60 hpf, PL cells migrate along the arterial intersegmental vessels dorsally to contribute to the formation of the dorsal longitudinal lymphatic vessel or ventrally to contribute to the formation of the TD between the DA and PCV. On the basis of this study, lymphatic formation defects upon *rasgrp1* knockdown were unlikely due to a secondary

effect of alterations in blood vascular development. In Ras-GRP1-MO-injected embryos, the differentiation of PCV and DA occurred normally, as revealed by properly formed venous-arterial-specific markers (Fig. 4). In addition, the formation of the PCV, DA, and primary ISVs were all unaltered by RasGRP1 knockdown (Fig. 4). Our observation is consistent with previous studies demonstrating that disruption of arterial development in zebrafish cannot impair lymphangiogenic sprouting nor cause TD defects (9, 31).

Although we found that Ras/ERK signaling is implicated in RasGRP1-mediated lymphangiogenesis (Fig. 6), the molecular details underlying the regulation of lymphatic development by RasGRP1 remains to be determined in the future. As RasGRP1 expression was detected in the PCV from 24–40 hpf, at a time when the secondary sprouts occurs, we speculate that Ras-GRP1-mediated Ras/ERK signaling may directly induce venous endothelial cells to form lymphangiogenic sprouts. As we found that knockdown both *flt4* and *rasgrp1* has a synergistic effect on the defects of lymphatic development (Fig. 7), we propose that RasGRP1 may function downstream of Flt4 to activate Ras proteins, thereby regulating lymphangiogenic sprout formation in zebrafish. It is also possible that Ras activation by RasGRP1 is implicated in the expression of Flt4, which in turn modulates lymphangiogenesis, as supported by a recent report showing that Ras proteins are involved in Flt4 expression and lymphatic development in mice (26). Another possibility is that the lymphangiogenic sprout formation is due to the inhibition of venous angiogenic sprouts by RasGRP1-mediated Ras/ERK signaling, similar to a model proposed to explain the segregation and generation of the DA and PCV in early vascular development in zebrafish embryos (32). It is also noteworthy that Ras/ERK signaling is required for both angiogenesis and lymphangiogenesis. Intriguingly, injection of RasGRP1-MO at a low dose only affects development of lymphatic system but not angiogenesis (Fig. 4). We speculate that the subtle change of the Ras/ERK signaling pathway mediated by RasGRP1 may dictate whether angiogenesis or lymphangiogenesis is affected. Nevertheless, the finding that RasGRP1 is implicated in lymphangiogenesis not only reveals a new physiological function for RasGRP1 but also paves the way for future studies to unravel the unique role of RasGRP1-mediated Ras/ERK signaling in lymphatic development.

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