Noncanonical protease-activated receptor 1 regulates lymphatic differentiation in zebrafish

Highlights

The Mmp13b-Par1-Gnai2a axis regulates lymphatic differentiation in zebrafish

Par1 mutant showed decreased prox1a expression in parachordal lymphangioblasts

Par1 promotes flt4 expression in the posterior cardinal vein of zebrafish embryos
Noncanonical protease-activated receptor 1 regulates lymphatic differentiation in zebrafish

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SUMMARY
The differentiation of lymphatic progenitors is a crucial step in lymphangiogenesis. However, its underlying mechanism remains unclear. Here, we found that noncanonical protease-activated receptor 1 (par1) regulates the differentiation of lymphatic progenitors in zebrafish embryos. Loss of par1 function impaired lymphatic differentiation by downregulating prox1a expression in parachordal lymphangioblasts and caused compromised thoracic duct formation in zebrafish. Meanwhile, the G protein gna12a, a par1 downstream effector, was selectively required for lymphatic development in zebrafish, and its mutation mimicked the lymphatic phenotype observed in par1 mutants. Interestingly, mmp13, but not thrombin, was required for lymphatic development in zebrafish. Furthermore, analyses of genetic interactions confirmed that mmp13b serves as a par1 upstream protease to regulate lymphatic development in zebrafish embryos. Mechanistically, par1 promotes flt4 expression and phospho-Erk1/2 activity in the posterior cardinal vein. Taken together, our findings highlight a function of par1 in the regulation of lymphatic differentiation in zebrafish embryos.

INTRODUCTION

The lymphatic system is a complex vasculature that originates from a preexisting embryonic vein. It has crucial functions in maintaining the interstitial fluid balance and retrieving water and macromolecules, taking up lipids, and also providing the major conduit for immune cells to take part in the immune surveillance system (Alitalo, 2011). Malformation of the lymphatic system can lead to many pathologies, such as tissue fluid accumulation, edema or lymphedema, cancer cell dissemination, and inflammation (Schulte-Merker et al., 2011).

During the development of the lymphatic system of mice, a sub-population of venous endothelial cells (VECs) expresses the transcription factor prosper homeobox protein 1 (PROX1) and differentiates into lymphatic progenitors in the cardinal vein (CV) at embryonic day 9.5 (E9.5) (Wigle et al., 2002; Wigle and Oliver, 1999). Ablation of prox1 in mice prevents the development of lymphatic vessels, and the ectopic expression of prox1 in endothelial cells (ECs) of blood vasculature upregulates lymphatic endothelial cell (LEC) markers (Kim et al., 2010; Wigle and Oliver, 1999). These studies suggest that prox1 is both necessary and sufficient to induce LEC fate (Hong et al., 2002; Wigle et al., 2002). In zebrafish embryos, lymphatic progenitors are induced within the ventral posterior cardinal vein (PCV) at an early stage, approximately 26 h postfertilization (hpf) (Nicenboim et al., 2015). The LECs show prox1a expression and move away to vacate the PCV in response to the sprouting process (Koltowska et al., 2015). These lymphatic progenitors migrate to the horizontal myoseptum (HM) region to form parachordal lymphangioblasts (PLs), a pool of lymphatic precursor cells, at about 48 hpf. Afterward, the PLs migrate in two directions, ventrally to form the thoracic duct (TD) and dorsally to form the dorsal longitudinal lymphatic vessel (DLLV); this is completed at around 5 days postfertilization (dpf) (Cha et al., 2012). Maternal and zygotic prox1a mutant leads to defective lymphatic development in zebrafish embryos, indicating its conserved role in the lymphatic vasculature. Recent studies have shown that a crucial role of Vegfc-Flt4 and its downstream effector Erk1/2 involves the induction of prox1a expression and LEC sprouting in zebrafish trunk (Koltowska et al., 2015; Shin et al., 2016).
Figure 1. *par1* mutant zebrafish embryos show defective TD formation

(A) WISH of *par1* gene expression in zebrafish embryos at 26 hpf. The white arrowhead indicates the expression of the *par1* gene in the PCV.

(B) Brightfield lateral views of sibling and *par1* homozygous mutant zebrafish embryos at 5 dpf. Scale bars: 1 mm.

| 5 dpf, Tg(*flila:EGFP*) | sibling | *par1* mutant |
|--------------------------|---------|---------------|

| 5 dpf, Tg(*flila:eap:dsRed;flila:EGFP*) | sibling | *par1* mutant |

| | | |
|---|---|---|
| ISV | ISV | |
| PCV | PCV | |
| TD | TD | |

| | | |
|---|---|---|
| Normal | 33 | 10 |
| Partial | 7 | 30 |
| Absent | 0 | 2 |

![Graph](image)

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**Figure 1.** *par1* mutant zebrafish embryos show defective TD formation

(A) WISH of *par1* gene expression in zebrafish embryos at 26 hpf. The white arrowhead indicates the expression of the *par1* gene in the PCV.

(B) Brightfield lateral views of sibling and *par1* homozygous mutant zebrafish embryos at 5 dpf. Scale bars: 1 mm.
Protease-activated receptor 1 (PAR1) is a G-protein-coupled receptor (GPCR), more specifically, a thrombin receptor (F2r) that plays a critical role in vascular biology (Alberelli and De Candia, 2014; Coughlin, 2005). It is activated through cleavage of the N-terminal exodomain by the serine protease thrombin (F2) at a canonical site (Vu et al., 1991). However, other PAR1 upstream proteases have been found to activate PAR1 (Alberelli and De Candia, 2014). For example, matrix metalloproteases (MMPs), especially MMP1 and MMP13, can also cleave and activate PAR1 at a noncanonical site, which leads to a signaling pattern that is distinct from that seen with F2 (Austin et al., 2013; Galt et al., 2002; Jaffré et al., 2012; Trivedi et al., 2009). Once irreversibly cleaved and activated by different proteases at either canonical or noncanonical sites, PAR1 can couple to and activate multiple heterotrimeric G protein subtypes including G12/13, G11/αq, and G13. This results in a multitude of cellular signaling events including the activation of MAPK/ERK signaling (Soh et al., 2010; Zhao et al., 2014). Par1 has been reported to play a pivotal role in hematopoiesis (Yue et al., 2010) and cardiovascular development in zebrafish (Ellertsdottir et al., 2012). Although par1 is enriched in the PCV of zebrafish embryos at 1 dpf (Xu et al., 2011), its role within it remains unknown.

Here, we report that the Mmp13b-Par1-Gnai2a axis regulates the differentiation of lymphatic progenitors in zebrafish embryos. Mechanistically, we show that par1 promotes Erk1/2 activity and the expression of the lymphatic fate marker prox1a by regulatingflt4 expression.

RESULTS

par1 is required for trunk lymphatic development in zebrafish embryos

PCV acts as a source of trunk lymphatic progenitors in zebrafish embryos (Yaniv et al., 2006). Interestingly, our results from whole-mount in situ hybridization (WISH) showed that par1 is highly expressed in the PCV region of zebrafish embryos at 26 hpf (Figure 1A), consistent with a previous report (Xu et al., 2011). Hence, par1 could be involved in lymphatic development during embryogenesis in zebrafish. To investigate this, we successfully generated a zebrafish mutant of par1 harboring a 103 bp deletion within its exon 2 using CRISPR/Cas9 technology (Figure 1B). We found that F2-generation embryos of the mutants seemed to be normal without any severe defects (Figure 1C). However, at 5 dpf, they showed impaired TD formation (Figures 1D and 1F). In siblings, only 6% of somites lacked TDs (Figures 1D and 1F). In contrast, nearly 25% of somites in par1 zebrafish mutants lacked them (Figures 1D and 1F). In addition, mutants had an average of only 11.2 number of LEC nuclei/6 somites (Figures 1E and 1G), significantly less than 17.5 found in sibling embryos (Figures 1E and 1G).

Taken together, these results confirmed that par1 is required for TD formation, which implies that it is involved in the development of the lymphatic trunk during zebrafish embryogenesis.

par1 regulates the differentiation of lymphatic progenitors in zebrafish

During the lymphatic development of zebrafish, Prox1a-positive endothelial cells sprout dorsally from the PCV at 30 hpf and form PLs as a pool of lymphatic progenitors at 48 hpf. At the same time, VECs sprout to form the venous intersegmental vessel (vISV) in parallel, which then fuses with the arterial intersegmental vessel (aISV) to establish a circulatory network with alternating arterial and venous connections (Mulligan and Weinstein, 2014; Semo et al., 2016). To determine whether par1 is involved in lymphatic differentiation,
we first performed a whole embryo immunostaining assay to examine expression of Prox1a in PLs of Tg(fli1a:EGFP) line at 54 hpf. Compared with sibling embryos, par1 zebrafish mutants showed a comparable reduction of prox1a expression in PLs (Figures 2A and 2B). Concomitant with the role of par1 in regulating prox1a expression in zebrafish embryos, we knocked down the expression of PAR1 in human dermal lymphatic endothelial cells (HDLECs) using an siRNA method. Interestingly, the expression of PROX1 significantly decreased not only in terms of mRNA levels (Figure 2C) but also in terms of protein levels (Figure 2D). These results suggest a conserved role of PAR1 in regulating PROX1 expression in vivo and in vitro.

Figure 2. par1 is required for lymphatic differentiation in zebrafish embryos

(A) Immunostaining of prospero homeobox protein-1a (Prox1a) in Tg(fli1a:EGFP) siblings and par1 homozygous mutants at 54 hpf. White arrowheads indicate the presence of parachordal lymphangioblasts (PLs) sprouting in each somite; blue arrowheads with white dashed lines represent positive Prox1a staining in PLs. Scale bars: 100 μm.

(B) Prox1a-positive PLs in siblings (n = 17 embryos) and par1 homozygous mutants (n = 22 embryos) at 54 hpf; 7 somites/embryos were used for quantification. Table shows the number of prox1a-positive segment per 7 somites in each embryo.

(C) Relative mRNA expression of PROX1 and PAR1 in human dermal lymphatic endothelial cells (HDLECs) after ctr-siRNA (control) and PAR1-siRNA transfection.

(D) Western blot analysis of PROX1 and PAR1 expression upon PAR1 knockdown in HDLECs. In (C and D), values represent means ± SEMs. *p ≤ 0.01, **p ≤ 0.001 in the Student’s t test.
lymphatic vessels. Sibling showed almost 100% venous and lymphatic sprouting from the PCV at 36 hpf (Figures S1A and S1B), whereas the par1 mutants showed only about 80% at the same stage (Figures S1A and S1B). Together, these results demonstrate that par1 is required for the differentiation of lymphatic progenitors in zebrafish embryos.

A number of different guanine-nucleotide-binding G-protein α-subunits function as downstream effectors of PAR1, including members of the G1, Gq/11, and G12/13 sub-families (Soh et al., 2010; Zhao et al., 2014). In seeking an alternative G protein involved in par1-mediated lymphangiogenesis in zebrafish embryos, we first performed quantitative real-time PCR (qPCR) analysis of HDLECs using seven G proteins from different subclasses including GNAQ, GNA11, GNA12, GNA13, GNAI1, GNAI2, and GNAI3. We found that only GNA11, GNA13, and GNAI2 had a relatively high mRNA expression level (Figure 3A). Then, we knocked down the expression of these three genes in HDLECs using an siRNA method and validated the expression level of PROX1. Surprisingly, the results showed that only knockdown of GNAI2 caused a significant decrease in PROX1 expression in terms of both mRNA (Figure 3B) and protein levels (Figure 3C). This indicates that GNAI2 is more likely to be involved in regulating lymphatic differentiation than other G proteins.

Next, we analyzed the expression pattern of gnai2 homologous genes (gnai2a and gnai2b) in zebrafish embryos via WISH. The results showed that gnai2a, but not gnai2b, is highly expressed in the PCV region in which the lymphatic trunk originated at 26 hpf (Figure 3D). To support this result, we then carried out knockdown assays by injecting gnai1a MO, gnai1b MO, gnai13a MO, gnai13b MO, as well as gnai2a MO into embryos in the one-cell stage. We inspected TD formation at 5 dpf in each respective zebrafish morphant. Interestingly, we discovered that only gnai2a morphants exhibited defective TD formation, with more than 50% of somites lacking TDs (Figures 3E and 3F).

**Figure 3. gnai2a is selectively required for lymphatic development in zebrafish embryos**

(A) Relative mRNA expression of different G-protein-coupled receptors in HDLECs.

(B) Relative mRNA expression of PROX1 in HDLECs upon transfection with ctr-siRNA (control), GNA11-siRNA, GNA13-siRNA, and GNAI2-siRNA.

(C) Western blot analysis of PROX1 and GNAI2 expression upon GNAI2 knockdown in HDLECs.

(D) WISH of gnai2a gene expression (upper) and gnai2b gene expression (below) at 26 hpf in zebrafish embryos. The white arrowhead indicates the PCV area.

(E) Confocal images showing TD formation in Tg(fli1a:EGFP) injected with 4 ng control MO, 4 ng gnai2a MO, 4 ng gnai11a MO, 4 ng gnai11b MO, 4 ng gnai13a MO, and 4 ng gnai13b MO at 5 dpf. Scale bars: 100 μm.

(F) Percentage of somites lacking TD formation. For each group, 30 embryos were quantified, and 6 somites/embryo were used for quantification. In (A), (B), and (F), values represent means ± SEMs. *p < 0.001; ns, not significant in Student’s t test.

gnai2a is selectively required for trunk lymphatic development in zebrafish embryos

A number of different guanine-nucleotide-binding G-protein α-subunits function as downstream effectors of PAR1, including members of the G1, Gq/11, and G12/13 sub-families (Soh et al., 2010; Zhao et al., 2014). In seeking an alternative G protein involved in par1-mediated lymphangiogenesis in zebrafish embryos, we first performed quantitative real-time PCR (qPCR) analysis of HDLECs using seven G proteins from different subclasses including GNAQ, GNA11, GNA12, GNA13, GNAI1, GNAI2, and GNAI3. We found that only GNA11, GNA13, and GNAI2 had a relatively high mRNA expression level (Figure 3A). Then, we knocked down the expression of these three genes in HDLECs using an siRNA method and validated the expression level of PROX1. Surprisingly, the results showed that only knockdown of GNAI2 caused a significant decrease in PROX1 expression in terms of both mRNA (Figure 3B) and protein levels (Figure 3C). This indicates that GNAI2 is more likely to be involved in regulating lymphatic differentiation than other G proteins.
Figure 4. *gnai2a* mutant mimics the lymphatic phenotypes observed in the *par1* mutant

(A) Confocal images showing TD formation in Tg(fli1a:EGFP) siblings and *gnai2a* homozygous mutants at 5 dpf. Blue arrowheads indicate TD formation in each somite; yellow asterisks represent the absence of TD formation in each somite. Scale bars: 100 µm.

(B) Confocal images showing LECs nuclear numbers in the TD tube of siblings and *gnai2a* homozygous mutants in the Tg(fli1aep:dsRed;fli1:nEGFP) line at 5 dpf. White circles indicate the presence of LECs nuclear numbers in the TD tube. Scale bars: 100 µm.

(C) Immunostaining of Prox1a of siblings and *gnai2a* homozygous mutants in the Tg(fli1a:EGFP) line at 54 hpf. White arrowheads indicate positive Prox1a staining in PLs. Scale bars: 100 µm.

(D) Percentage of somites lacking TD formation in siblings (n = 50) and *gnai2a* homozygous mutants (n = 60 embryos); 6 somites/embryos were used for quantification.

(E) LEC nuclear number in the TD tube of siblings (n = 32) and *gnai2a* homozygous mutants (n = 34 embryos); 6 somites/embryos were used for quantification.

(F) Prox1a expression in PLs of siblings (n = 25) and *gnai2a* homozygous mutants (n = 32).
and gna13b morphants showed normal TD formation (Figures 3E and 3F). These results indicate that gna2a is selectively required for lymphatic development in zebrafish embryos.

**gna2a recapitulates par1-mediated phenotypes during lymphatic development in zebrafish**

To investigate the role of gna2a during zebrafish lymphangiogenesis, we used CRISPR/Cas9 technology to obtain a gna2a mutant harboring a 68 bp deletion in its exon 3 (Figure S2A). The F2 generations of most mutants had normal phenotypes at 5 dpf (Figure S2B). Next, we examined TD formation at 5 dpf during embryogenesis. As shown in Figure 4, nearly 30% of somites lacked TDs in the mutants, significantly more than sibling embryos. In addition, we assessed the number of LEC nuclei/6 somites in TD tubes at 5 dpf using the transgenic zebrafish line Tg(fliaep:dsRed;fli1a:nEGFP). The mutants had notably decreased numbers, with an average of 11.7 compared with an average of 18.2 in sibling embryos (Figures 4Ba and 4E).

To evaluate whether gna2a is required to regulate lymphatic differentiation, we conducted an immunostaining assay using anti-Prox1 antibody at 54 hpf in Tg(fli1a:EGFP) embryos. We found that F2 generations of mutants had fewer prox1a-positive PLs than sibling embryos (Figures 4C and 4F). Meanwhile, we found that knockdown of gna2a caused a partial failure in the formation of lympho-venous sprouting at 36 hpf, compared with control embryos with normal sprouting (Figures S3A and S3B). This suggests that gna2a is required for lymphatic differentiation in zebrafish.

To further validate that gna2a is the downstream effector of par1 in this process, we cross-bred par1 heterozygous mutant and gna2a heterozygous mutant using the Tg(fli1a:EGFP) line and Tg(fli1aep:dsRed;fli1:nEGFP) line. We found that wild-type embryos, par1 heterozygous mutants, gna2a heterozygous mutants, and cross-bred par1-gna2a heterozygous mutants had normal morphologies (Figure S4E) and did not display a comparable difference in TD formation (Figures S4A and S4C). However, interestingly, we found that the average number of LEC nuclei/6 somites in the cross-bred mutants was 13.2, compared with 17.6 in wild-type embryos and about 15 in each par1 heterozygous mutant and gna2a heterozygous mutant (Figures S4B and S4D). This indicates that there is a genetic interaction between par1 and gna2a in cross-bred par1-gna2a heterozygous zebrafish mutants, especially at the cellular level.

Taken together, these results show that the gna2a zebrafish mutant mimics the phenotypes of par1 zebrafish mutants, indicating that gna2a is the downstream effector of par1 in the regulation of zebrafish lymphangiogenesis.

**Thrombin is not involved in zebrafish lymphangiogenesis**

F2 was the first ligand to be reported to have the ability to cleave and activate PAR1 at a canonical site (Vu et al., 1991). To elucidate whether F2 is required for lymphatic development in zebrafish embryos, we injected F2 MO into embryos (4 ng/embryo) in the one-cell stage. At 5 dpf, we analyzed TD formation and unexpectedly found that both control and F2 morphants had normal TD formation (Figures S5A and S5C). To further validate this result, we treated zebrafish embryos with SCH79797, a specific PAR1 antagonist that blocks the interaction between F2 and PAR1. Consistent with the above results, both treated and vehicle control embryos exhibited normal TD formation at 5 dpf (Figures S5B and S5D), suggesting that par1 is independent of F2 in regulating lymphatic development in zebrafish. These results clearly indicate that F2 is not required for lymphatic development in zebrafish.

**Noncanonical mmp13b is required for lymphatic development in zebrafish**

Several proteases cleave and activate PAR1, including F2, plasmin, activated protein C, thrombocytin, factor Xa, factor VIIa, tryptase, trypsin, MMPs, and so on (Zhao et al., 2014). To search for candidate proteases involved in PAR1 regulation of lymphangiogenesis in zebrafish, we first conducted qPCR analyses of HDLECs and observed the relative mRNA expression of these proteases. Interestingly, MMP1 showed the highest relative mRNA expression (Figure 5A). Next, we transfected MMP1-siRNA into HDLECs and evaluated the expression of PROX1. Interestingly, knockdown of MMP1 caused a significant reduction of PROX1 mRNA expression, suggesting that MMP1 may be involved in lymphangiogenesis (Figure 5B).
Figure 5. **mmp13b** mutant mimics the lymphatic phenotypes observed in the **par1** mutant
(A) Relative mRNA expression of different **PAR1** proteases in HDLECs.
(B) Relative mRNA expression of **PROX1** and **MMP1** in HDLECs after **ctr-siRNA** (control) and **MMP1-siRNA** transfection.
(C) WISH of **mmp13b** gene expression in zebrafish at 26 hpf. The white arrowhead indicates the expression of the **mmp13b** gene in the PCV area.
In the human genome, there are three collagenases (MMP1, MMP8, and MMP13). However, two of them (Mmp1 and Mmp8) have no zebrafish homolog, and only MMP13 is present in duplicate (Mmp13a and Mmp13b) in the zebrafish genome (Wyatt et al., 2009). mmp13a is expressed in myeloid lineage cells but not in the vein (Qian et al., 2005). Therefore, we speculated that mmp13b could replace the function of the human MMP1 gene to regulate par1-mediated lymphatic development in zebrafish embryos. We used WISH to observe the expression patterns of mmp13b during zebrafish embryogenesis. Interestingly, we found that it is highly expressed in the PCV region at 26 hpf, suggesting that it is involved in lymphatics (Figure S5C). Next, we generated a zebrafish mmp13b mutant using CRISPR/Cas9 technology and obtained a stable mutant harboring a 7 bp deletion in its exon 4 (Figure S6). To validate the role of mmp13b in regulating lymphangiogenesis, we examined TD formation in the F2 generation of embryos. As observed in the par1 zebrafish mutant, mmp13b mutant embryos showed nearly 25% of somites lacking TD at 5 dpf, compared with siblings with normal TD formation (Figures S5D and S5G). We also checked the number of LEC nuclei in TD tube with transgenic zebrafish line Tg(fli1aep:dsRed;fli1a:nEGFP) at 5 dpf and found that the mutant had significantly fewer numbers than sibling embryos (Figures S5E and S5H). Furthermore, immunostaining assays of Prox1a expression in PLs at 54 hpf indicated that mutants also showed significantly reduced Prox1a expression compared with siblings (Figures S5F and S5I). Together, these data demonstrate that mmp13b is required for lymphatic development in zebrafish.

**Overexpression of cleaved par1 recovers the phenotypes of mmp13b mutant in zebrafish lymphangiogenesis**

As observed with the tethered ligand sequence (SFLLRN) in human PAR1, zebrafish Par1 also harbors a putative tethered ligand sequence (SFSGFL) within the extracellular N-terminal region (Kim et al., 2009; Xu et al., 2011), which can induce thrombocyte activation (Kim et al., 2009). Meanwhile, MMP-13 can cleave PAR1 at the S_{22}F_{LLRN} site, and cleaved PAR1 changes its conformation and activates downstream ERK1/2 signaling in cardiac cells (Jaffré et al., 2012). To confirm the function of mmp13b as an upstream par1 protease that regulates lymphatic development in zebrafish, we generated a zebrafish transgenic line, Tg(lyve1b:cleaved-par1-P2A-mCherry), in which cleaved Par1 at the S_{22}F_{SGFL} site (named cleaved-par1) was fused into cleaved-par1-P2A-mCherry and was driven by the venous and lymphatic promoter lyve1b. Then we crossed mmp13b mutant with the Tg(lyve1b:cleaved-par1-P2A-mCherry) line for rescue experiments. We examined TD formation at 5 dpf both for the mmp13b zebrafish mutant and siblings in these two transgenic lines. Compared with the normal TD formation of siblings in the Tg(lyve1b:mCherry) line at 5 dpf, the mmp13b zebrafish mutant lacked TD at 5 dpf (Figures 6A and 6B). Meanwhile, overexpression of par1 under the lyve1b promoter did not induce ectopic TD formation, and overexpression showed a normal TD morphology (Figures 6A and 6B). However, the mutant in the Tg(lyve1b:cleaved-par1-P2A-mCherry) background showed normal TD formation (Figures 6A and 6B), indicating that the overexpression of par1 in the mmp13b zebrafish mutant can recover the defect phenotype. Hence, mmp13b could serve as an upstream par1 protease that regulates lymphatic development in zebrafish embryos.

**par1 promotes Erk1/2 activity and flt4 expression in zebrafish embryos**

Vegfc and its receptor Flt4 activate p-Erk1/2 by inducing prox1a expression, ultimately regulating the differentiation of lymphatic progenitor cells in zebrafish embryos (Kołowska et al., 2015; Küchler et al., 2006;
Based on our above results, we propose that 
par1 may promote Vegfc/Flt4/Erk signaling activity to regulate lymphangiogenesis in zebrafish embryos. To test this hypothesis, we performed a whole-embryo p-Erk1/2 immunostaining assay using the F2 generations of 
Tg(fli1a:EGFP) embryos. At 28 hpf, when lymphatic progenitors start migrating dorsally from the PCV, we observed clear p-Erk1/2 staining in the PCV of sibling embryos (Figures 7A and 7B). However, there was significantly less staining in the PCV of 
par1 zebrafish mutants (Figures 7A and 7B). Next, to confirm whether 
PAR1 promotes VEGFC-induced p-ERK1/2 activation, we performed 
PAR1 knockdown experiments in cultured HDLECs and found that knockdown of PAR1 caused a significant decrease in VEGFR3 mRNA level (Figure 7C). We then evaluated the effect of PAR1 on VEGFC-induced phosphor-ERK1/2 activity in HDLECs in vitro. In HDLECs transfected with control-siRNA (ctr-siRNA), a significant increase in ERK1/2 phosphorylation was detected at 15 min. In contrast, HDLECs transfected with 
PAR1-siRNA showed a significant decrease in p-ERK1/2 levels (Figure 7D). Meanwhile, knockdown of PAR1 also caused a compromised VEGFR3 expression at the protein level (Figure 7D).

In parallel, we also examined the expression of 
flt4 during zebrafish embryogenesis. The results from qPCR analysis showed that loss of 
par1 function decreased 
flt4 mRNA levels in zebrafish embryos (Figure 7E). Meanwhile, 
par1 mutants clearly showed a reduction in 
flt4 expression at 28 hpf, compared with sibling in the same stage (Figure 7F). Transcription factor 
hhex has been reported to regulate 
flt4 mRNA levels in the PCV during the lymphatic development of zebrafish (Gauvrit et al., 2018). To determine whether 
par1 regulates 
hhex expression in zebrafish embryos, we performed WISH tests. 
par1 mutants showed decreased 
hhex expression in the PCV at 28 hpf, compared with siblings (Figure 7F). Together, these results demonstrate that 
par1 promotes 
flt4 expression during lymphatic development in zebrafish.

DISCUSSION
The PAR family, especially 
PAR1, is a critical mediator of vascular hemostasis, thrombosis, and inflammation (Alberelli and De Candia, 2014). Here, we found compelling evidence that the noncanonical
Figure 7. Loss of par1 function decreases p-Erk1/2 activity and flt4 expression in the PCV of zebrafish embryos

(A) Immunostaining of phosphoErk1/2 (p-Erk1/2) activity in Tg(fli1a:EGFP) siblings and par1 homozygous mutants at 28 hpf. Blue arrowheads with white dashed lines indicate positive p-Erk1/2 staining; DA and PCV (in brackets) area are noted. Scale bars: 100 μm.

(B) Western blot analysis of VEGFR3, p-ERK1/2, ERK1/2, PAR1, and β-actin expression in siblings and par1 homozygous mutants under VEGF-C stimulation (0, 15, 30, and 60 min).

(C) Relative mRNA expression of PAR1 and VEGFR3 under control (ctrl-siRNA) and PAR1-siRNA conditions.

(D) Relative mRNA expression of par1 and flt4 in wild type (WT) and par1 mutant conditions.
Figure 7. Continued
(B) Quantification of p-Erk1/2 expression staining in the PCV of siblings (n = 15 embryos) and par1 mutants (n = 15 embryos); 8 somites/embryos were used for quantification.
(C) Relative mRNA expression of VEGFR3 in HDLECs after ctr-siRNA (control) and PAR1-siRNA transfection.
(D) Western blot analysis of p-ERK1/2 activity in HDLECs transfected with ctr-siRNA or PAR1-siRNA, followed by VEGFC treatment.
(E) Relative mRNA expression of the flt4 gene in siblings and par1 homozygous zebrafish embryo mutants.
(F) WISH of flt4 gene expression at 28 hpf in wild-type and par1 homozygous mutant embryos. The white arrowhead indicates the PCV area.
(G) WISH of hhex gene expression at 28 hpf in wild-type and par1 mutant embryos. The white arrowhead indicates the PCV area. In (B, C, and, E), values represent means ± SEMs. *p ≤ 0.05, **p ≤ 0.001, ***p ≤ 0.0001 in the Student’s t test.

Mmp13b-Par1-Gna2a axis regulates the differentiation of trunk lymphatic progenitors by promoting flt4 expression in the PCV in the early stage (Figure 8).

In zebrafish embryos, trunk lymphatic progenitors originate from the PCV (Yaniv et al., 2006), suggesting that genes enriched in the PCV may be involved in lymphangiogenesis in zebrafish. We found several lines of evidence that par1 regulates lymphatic differentiation in zebrafish. First, par1 is highly enriched in the PCV at 26 hpf. Second, par1 regulates prox1 expression in vitro and in vivo. Third, par1 mutant shows decreased flt4 expression and compromised phosphorylation-Erk1/2 activity in the PCV, both of which are crucial for lymphatic differentiation in zebrafish embryos (Koltowska et al., 2015; Shin et al., 2016). However, both defective venous-lymphatic sprouting and subsequent compromised TD formation are mild in the par1 mutant, whereas par1 homozygous mutants showed clear reduction in prox1a expression in PLs at 54 hpf and in the nucleus number of LECs in TD tubes at 5 dpf. These results suggest that par1 plays an important role in lymphatic differentiation rather than in lymphatic migration in zebrafish embryos. Consistent with this, prox1a mutant zebrafish embryos show normal TD formation (van Impel et al., 2014), whereas maternal and zygotic prox1a mutant zebrafish show a significant decrease in number of LEC nuclei (Koltowska et al., 2015).

Several G proteins (including GNA12/13, GNAQ/11, and GANI1/2/3) function as downstream effectors of PAR1 (Zhao et al., 2014). Here, we demonstrated that gna2a is selectively required for par1-mediated lymphatic development in zebrafish embryos. Furthermore, gna2a mutants mimicked the phenotypes observed in par1 mutants in terms of lymphatic development. We also performed genetic interaction analyses by cross-breeding par1 heterozygous zebrafish mutant and gna2a heterozygous zebrafish mutants. The morphologies of the embryos did not show any significant differences at 5 dpf, compared with wild-type embryos. However, nucleus counting at 5 dpf indicated significantly fewer number of LEC nuclei in the par1-gna2a heterozygous mutant embryos. This indicates that there is a genetic interaction at the cellular level between par1 and gna2a.

Several PAR1 upstream proteases have been identified, such as F2, plasmin, factor Xa, factor VIIa, trypsin, MMPs, and others (Zhao et al., 2014). Surprisingly, canonical F2 was not involved in lymphatic development in zebrafish embryos. Although human MMP1 regulates PROX1 expression in HDLECs in vitro, our results strongly suggest that mmp13 serves as an upstream protease of par1 that regulates lymphatic development in zebrafish. In mice, there is no mmp1 gene, and MMP13 is thought to play the role of human MMP1, cleaving and activating PAR1 at the conserved S42↓↓FLLRN site (Jaffré et al., 2012). In zebrafish, two orthologs of MMP13 (Mmp13a, Mmp13b) are present (Wyatt et al., 2009). Mmp13a is expressed in myeloid lineage cells but not in the vein (Qian et al., 2005). Interestingly, we found that mmp13b is highly expressed in the PCV region of zebrafish embryos at 26 hpf. Furthermore, we found that zebrafish mmp13b mutants perfectly mimicked the phenotypes of par1 zebrafish mutants. Further analysis also revealed that the overexpression of cleaved par1 in mmp13b zebrafish mutants rescued the defective TD phenotype. These results suggest that mmp13b acts upstream of par1 to regulate lymphatic development in zebrafish embryos.

During LEC progenitors differentiation, these Prox1-positive cells migrate dorsally out of PCV, sprouting in response to signaling induced by VEGFC, through its receptor VEGFR3 or FLT4 to form the first lymphatic vessels (Haiko et al., 2008; Hogan et al., 2009a, 2009b; Karkkainen et al., 2004; Küchler et al., 2006). Disruption of vegfc or flt4 causes a severe defect in the lymphatic development of zebrafish and mice (Hogan et al., 2009a, 2009b; Karkkainen et al., 2004; Küchler et al., 2006; Villefranc et al., 2013; Yaniv et al., 2006). Further, Vegfc/Flt4 signaling can induce prox1a expression through Erk1/2, therefore initiating the differentiation and sprouting of lymphatic endothelial cells in the lymphatic trunk of zebrafish (Shin et al., 2016).
In our study, we found several lines of evidence indicating that *par1* regulates Vegfc/*Flt4*/Erk1/2 signaling during lymphatic development in zebrafish. First, loss of the *par1* gene reduced *flt4* expression. Second, the *par1* zebrafish mutant showed decreased expression of *hhex*, an upstream transcription factor of *flt4* in zebrafish embryos (Gauvrit et al., 2018). Third, disruption of *par1* inhibited phospho-Erk1/2 activities in the PCV of zebrafish embryos and VEGFC-induced phospho-ERK1/2 activity in vitro. A high level of VEGFR3/FLT4 may be a crucial prerequisite for the sensitivity of LEC progenitors to the VEGF-C induction (Ducoli and Detmar, 2021). Consistent with this, our zebrafish *par1* mutant showed significantly less *prox1* expression and compromised *flt4* expression. In contrast, disruption of the *par1* gene caused a mild defect in venous-lymphatic sprouting and in subsequent TD formation. We speculate that high expression of *flt4* is much more sensitive to lymphatic differentiation than that to venous-lymphatic sprouting in zebrafish embryos. Previous document reported that *flt4* null mutant showed clear edema, whereas *flt4*Y1226/7A mutants have no edema (Shin et al., 2016). Further analysis revealed signaling through *flt4* Y1226/7 and ERK is required for trunk lymphatic development but not for facial lymphatic formation. This indicated that facial lymphatic vessels, but not the thoracic duct, are initially dispensable for lymphatic function in zebrafish embryos. In this study, we found that *par1* mutant, *mmp13b* mutant, and *gnai2a* mutant have no edema. It is possibility that *par1* signaling mainly regulated trunk lymphatic development in zebrafish embryos through *flt4* and Erk1/2 signaling, which supported the previous notion (Shin et al., 2016).

Taken together, our results uncover a mechanism in which the *mmp13b-par1-gnai2a* axis regulates the lymphangiogenic process in the lymphatic trunk of zebrafish by promoting Vegfc/*Flt4*/Erk1/2 signaling, which induces *prox1a* expression.

**Limitation of the study**

In our study, we generated three mutants and found that *mmmp13b-par1-gnai2a* axis regulates the differentiation of lymphatic progenitors in zebrafish embryos. Mechanistically, *par1* promotes *flt4* expression and phospho-Erk1/2 activity in the posterior cardinal vein. However, the mechanism linking the *mmp13b-par1-gnai2a* pathway to regulation of *flt4* would need to be further expanded.
STAR METHODS
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Supplemental information can be found online at https://doi.org/10.1016/j.isci.2021.103386.

ACKNOWLEDGMENTS
This study was supported by grants from the National Natural Science Foundation of China (31771599, 31971242), Chongqing Science and Technology Bureau (cstc2019jcyj-zdxmX0028), and the National Key Technology R&D Program of China (2016YFC1102305). The authors want to thank the assistance from the Chongqing Engineering Laboratory in Vascular Implants and the Public Experiment Center of State Bioindustrial Base (Chongqing), China.

AUTHOR CONTRIBUTIONS
Conceptualization, Y.W.; Methodology, D.L., M.A.R., X.Z., Y.M., X.Z., and L.W.; Investigation, Y.W., D.L., M.A.R., and X.Z.; Writing—Original Draft, Y.W., G.W., D.L., and M.A.R.; Writing—Review & Editing, Y.W., G.W., D.L., and M.A.R.; Funding Acquisition, Y.W. and G.W.; Resources, L.L., T.Z., and Y.L. Supervision, Y.W. and G.W. All authors approved the final version of the manuscript.

DECLARATION OF INTERESTS
The authors declare that there is no conflict of interest.

Received: March 30, 2021
Revised: August 26, 2021
Accepted: October 26, 2021
Published: November 19, 2021

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Recombinant anti-PROX1 antibody | GeneTex | Cat#GTX128354;RRID:AB_2893482 |
| F2R rabbit pAb      | ABlonal | Cat#A5641;RRID:AB_2766401 |
| GNAI2 rabbit pAb    | ABlonal | Cat#A7676;RRID:AB_2769633 |
| Anti-alpha tubulin antibody | Abcam | Cat#ab18251;RRID:AB_2210057 |
| GAPDH (D16H11) XP® Rabbit mAb | Cell Signaling Technology | Cat#S1774;RRID:AB_10622025 |
| p44/42 MAPK (Erk1/2) (137FS) Rabbit mAb | Cell Signaling Technology | Cat#4695;RRID:AB_390779 |
| Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP® Rabbit mAb | Cell Signaling Technology | Cat#4370;RRID:AB_2315112 |
| B-actin             | ZEN BIO | Cat#380624;RRID:AB_2893488 |
| Goat anti-Mouse IgG (H&L) (HRP conjugate) | ZEN BIO | Cat# 511103;RRID:AB_2893489 |
| Goat anti-rabbit IgG (H+L) secondary antibody SSS | ThermoFisher Scientific | Cat#A21434;RRID:AB_2535855 |
| **Chemicals, peptides, and recombinant proteins** |        |            |
| TritonX-100         | Sigma  | T9284      |
| Bovine Serum Albumin| Sigma  | A9647      |
| 4x protein loading buffer | Li-cor | 928-40004 |
| PTU                 | Sigma  | 2954-52-1  |
| Tricaine            | Sigma  | 886-86-2   |
| SCH77977 dihydrochloride | MCE | HY-14994  |
| BM Purple AP        | Roche  | 11442074001 |
| RNA from yeast      | Roche  | 1010923001 |
| Proteinase K        | MERCK  | 39450-01-6 |
| Recombinant human VEGF-C (Cys156Ser) protein | R&D Systems | Cat# 752-VC-025 |
| **Critical commercial assays** |        |            |
| Tol2 kit            | (Kwan et al., 2007) | N/A |
| LR Clonase II Plus Enzyme mix | Invitrogen | 12538200 |
| HiScribe T7 ARCA mRNA kit | NEB | #E20655 |
| DIG RNA Labeling Mix | Roche | 1127703910 |
| TB Green Fast RT-PCR Mix | TAKARA | RR430A |
| **Experimental models: Cell lines** |        |            |
| Human Primary Lymphatic Endothelial Cells | CellBiologics | H-6092 |
| **Experimental models: Organisms/strains** |        |            |
| Zebrafish: Tg(fli1a:EGFP)57 | (Jin, 2005) | N/A |
| Zebrafish: Tg(fli1a:eap:DsRedEX)533 | (Proulx et al., 2010) | N/A |
| Zebrafish: Tg(fli1a:nEGFP)47 | (Roman et al., 2002) | N/A |
| Zebrafish: Tg(kdrl:mCherry) | (Proulx et al., 2010) | N/A |
| Zebrafish: Tg[lyve1b:TopazYFP]1015 | This paper | N/A |
| **Oligonucleotides** |        |            |
| sgRNA targeting sequence: par1 : GGGGAAAGCGGCTTAGTTCGGG | This paper | N/A |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to the lead contact: Yeqi Wang (yeqi.wang@cqu.edu.cn).

Materials availability
Transgenic zebrafish lines and mutants generated in this study are available from lead contact upon request.

Data and code availability
Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request. This paper does not report original code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Zebrafish
Zebrafish (Danio rerio) embryos were incubated in Holtfreter’s solution at 28.5°C (Wang et al., 2011). The following transgenic fish lines were used: Tg(fli1a:EGFP)^1 (ref. (Jin, 2005)), Tg(fli1aep:DsRedEX)^m12 (ref.
(Proulx et al., 2010)), Tg(fli1a:nEGFP) (ref. (Roman et al., 2002)), Tg(kdrl:mCherry) (ref. (Proulx et al., 2010)), and Tg(lyve1b:TopazYFP) (ref. [34]), which were gifted by Prof. Anming Meng from Tsinghua University, China, and were generated according to a previous report (Okuda et al., 2012). All zebrafish maintenance and experiments were carried out in accordance with the guidelines approved by the Ethics Committee of Chongqing University.

METHOD DETAILS
Generation of the zebrafish transgenic line
To generate the construct for Tg(lyve1:mCherry) and Tg(lyve1:cleaved-par1-P2A-mCherry), we used Gateway-compatible vectors of the Tol2kit (Kwan et al., 2007). For venous and lymphatic expression, a 5.2 kb fragment of the zebrafish lyve1 promoter (Okuda et al., 2012) was subcloned into p5E-MCS. Zebrafish par1 cDNA, which lacks 87 bp within its N-terminal, was amplified by PCR. Then this par1 fragment, P2A sequence, and mCherry sequence were subcloned into pME-cleaved-par1-P2A-mCherry plasmid. The p5E-lyve1 plasmid was combined with pME-mCherry or pME-cleaved-par1-P2A-mCherry, the 3’ entry clone p3E-polyA, and the pDestTol2pA2 destination vector to create the pDest-lyve1:mCherry or pDest-lyve1:cleaved-par1-P2A-mCherry construct using LR Clonase II Plus Enzyme mix (Invitrogen, Cat#12538200). Embryos in the one-cell stage were injected with 25 ng/μL plasmid and 25 ng/μL Tol2 transposase RNA. Embryos injected with pTol2-lyve1:mCherry or pTol2-lyve1:cleaved-par1-P2A-mCherry were raised to adults and screened for founders.

Generation of zebrafish mutants using CRISPR/Cas9 technology
Zebrafish mutants were generated using CRISPR/Cas9 from Nanjing XinJia Medical Technology, Co., LTD, China. Briefly, the par1 zebrafish mutant was targeted to exon 2 of the par1 gene locus, with the target sequence 5’- GGGGGAAGCGGCTTTAGTTCGGG -3’; the gna12a zebrafish mutant was targeted to exon 3 of the gna12a gene locus, with the target sequence 5’-GTGCAAGCAGTATCGAGCTG-3’; and the mmp13b zebrafish mutant was targeted to exon 4 of the mmp13b gene locus, with the target sequence 5’-CCTCCTGGAATCGGCATTGG-3’. Cas9 mRNA (600 ng/μL) and par1, gna12a, and mmp13b gRNA (300 ng/μL) were co-injected into wild-type embryos in the one-cell stage. Founder (F0) were grown into adulthood and then were identified by genomic PCR and sequencing. Next, to confirm germline-transmitted mutations of par1, gna12a, and mmp13b, the identified F0 were crossed with wildtype fish to obtain heterozygous mutant zebrafish (F1). These were in-crossed to generate homozygous mutant embryos (F2) and used for the experimental analyses. Genotyping was carried out to distinguish between siblings (wild type), heterozygous mutants, and homozygous mutants after PCR amplification of target sites using caudal fin DNA (for adulthood) or whole embryo DNA (for embryonic stage); sequencing was used for verification. The primers for genotyping par1 zebrafish mutant, gna12a zebrafish mutant, and mmp13b zebrafish mutant are listed in Table S1.

Microinjection of MOs
All MOs were ordered from Gene Tools LIC. Embryos in the one-cell stage were injected with 4 ng anti-sense MO/embryo. TD formation of embryos at 5 dpf was observed by via confocal microscopy (Leica, SP8, Germany). The MO sequences used are listed in key resources table.

Antagonist exposure
A stock solution of SCH77977 was dissolved in DMSO to 10 mM, and a working solution was freshly prepared by diluting the stock solution to 100 nM with E3 embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, and 0.33 mM MgSO4). Then 100 ng of Tg(fli1a:EGFP) zebrafish embryos were collected and equally divided into two groups at random. One group was exposed to the SCH77977 working solution, and the other group was exposed to vehicle control. The solutions were used to treat embryos at 1 dpf and were changed once a day; dead embryos were removed using a stereoscopic microscope (Carl Zeiss, Jena, Germany). TD formation of zebrafish embryos at 5 dpf in both groups was observed via confocal microscopy (Leica, SP8, Germany).

Imaging
Zebrafish embryos were treated with 0.003% 1-phenyl-2-thiourea (PTU, Sigma) to prevent pigment formation from 24 hpf. And the embryos were mounted in 0.8% low-melting agarose (Sigma) from imaging. For
imaging, Leica SP8 microscopy was setting as following: magnification 25× water objective, 0.75× zoom in, and a step size of 1.5 μm.

**In situ hybridization**

WISH of zebrafish embryos was following the standard procedure. Stained embryos were mounted in 5% methylcellulose and glycerol, and the images were captured using a microscope (ZEISS Stemi 2000-C) (Li et al., 2012). The probe primers of specific genes are listed in Table S1.

**Whole mount immunostaining**

Live embryos were fixed in fresh 4% paraformaldehyde at 4°C overnight. The embryos were washed three times and dehydrated through a series of washings with methanol in PBST. Then they were placed in 100% methanol at -20°C overnight and re-dehydrated through a series of washings with methanol in PBST. The samples were gradually warmed in Tris buffer (150 mM Tris-HCl, pH 9.0), placed in a water bath from 37°C to 70°C, and then kept for 30 min. This was followed by gradually cooling to room temperature. Then the embryos were treated with Proteinase K (30 μg/mL) for 40 min for Prox1 staining or with Proteinase K (10 μg/mL) for 5 min for p-Erk1/2 staining at room temperature. Next, we blocked the embryos for 3–4 h at 4°C and then incubated them with the primary antibody (anti-Prox1 antibody, GeneTex: GTX128354; p-Erk1/2 antibody, Cell Signaling Technology: #4370) in 1:500 dilution buffer at 4°C overnight.

The embryos were washed and then incubated with goat anti-rabbit IgG (H+L) secondary antibody (Alexa 555-1:750 dilution in dilution buffer) for 1.5 h at room temperature under dark conditions. Finally, the embryos were washed in PBST-S and PBST at room temperature before being used for imaging.

**Cell culture and western blotting**

Human Primary Lymphatic Endothelial Cells (HDLECs) were obtained from CellBiologics and cultured with endothelial cell medium (ECM) and 10% fetal calf serum (FBS). All siRNAs and antibodies used in this study are listed in key resource table. Recombinant human VEGF-C (Cys156Ser) protein was purchased from R&D Systems (Catalogue: 752-VC-025). HDLECs were growth to 60-80% confluence and transfected with siRNA using RNAiMAX (Invitrogen). 72 hours after transfection, the HDLECs were serum starved for 8 hours, followed by 100 ng/ml VEGF-C stimulation. Then the HDLECs were harvested for Western blotted with standard protocol (Cui et al., 2014).

**Quantitative RT-PCR analysis**

Quantitative real-time PCR (qPCR) was performed with TB Green Fast RT-PCR Mix (TAKARA, RR430A). The qPCR primers are listed in Table S1.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

We used 6 somites/embryo, 6 somites/embryo, 8 ISVs/embryo, 7 somites/embryo, 7 somites/embryo, and 8 somites/embryo to quantify TD formation, LECs nuclear number, lympho-venous sprouting in the PCV, PL sprouting, Prox1 expression staining, and p-Erk1/2 expression staining in the PCV, respectively. All statistical analyses were done using GraphPad Prism software. The statistical significance of the difference between control and experimental groups was determined using the Student’s unpaired two-tailed t-test followed by one-way analysis of variance. Data are presented as mean ± SEM. P-values ≤ 0.05 were considered significant. ***<0.0001, **<0.001, *<0.01, ns>0.01.