1 **GATA2 controls lymphatic endothelial cell junctional integrity and lymphovenous valve**

2 **morphogenesis through *miR-126***

3

4 Md. Riaj Mahamud[1,2,#], Xin Geng[1,#], Yen-Chun Ho[1], Boksik Cha[1], Yuenhee Kim[3], Jing Ma[4],

5 Lijuan Chen[1], Greggory Myers[5], Sally Camper[5], Debbie Mustacich[6], Marlys Witte[6], Dongwon

6 Choi[7], Young-Kwon Hong[7], Hong Chen[8], Gaurav Varshney[9], James Douglas Engel[5],

7 Shusheng Wang[4], Tae-Hoon Kim[3], Kim-Chew Lim[5, #], R. Sathish Srinivasan[1,2, #,*]

8

9 [1] Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation,

10 Oklahoma City, Oklahoma, USA; [2] Department of Cell Biology, University of Oklahoma Health

11 Sciences Center, Oklahoma City, Oklahoma, USA; [3] Department of Biological Sciences and

12 Center for Systems Biology, The University of Texas at Dallas, Richardson, TX, USA; [4]

13 Department of Cell and Molecular Biology, Tulane University, New Orleans, Louisiana, USA; [5]

14 Department of Cell and Developmental Biology, University of Michigan Medical School, Ann

15 Arbor, Michigan, USA; [6] Lymphology Laboratories, University of Arizona, Tuscon, Arizona,

16 USA; [7] Keck School of Medicine, University of Southern California, Los Angeles, CA, USA; [8]

17 Vascular Biology Program, Boston Children's Hospital, Boston, MA, USA; [9] Functional and

18 Chemical Genomics Research Program, Oklahoma Medical Research Foundation, Oklahoma

19 City, Oklahoma, USA

20

21 [#] These authors contributed equally

22 *Correspondence: sathish-srinivasan@omrf.org.

23

24

1

1 **ABSTRACT**

2 Mutations in the transcription factor GATA2 cause lymphedema. GATA2 is necessary for the

3 development of lymphatic valves (LVs) and lymphovenous valves (LVVs), and for the

4 patterning of lymphatic vessels. Here, we report that GATA2 is not necessary for valvular

5 endothelial cell (VEC) differentiation. Instead, GATA2 is required for VEC maintenance and

6 morphogenesis. GATA2 is also necessary for the expression of cell junction molecules VE-

7 Cadherin and Claudin5 in lymphatic vessels. We identified *miR-126* as a target of GATA2, and

8 *miR-126*$^{-/-}$ embryos recapitulate the phenotypes of mice lacking GATA2. Primary human

9 lymphatic endothelial cells (HLECs) lacking GATA2 (GATA2$^{\Delta HLEC}$) have altered expression of

10 Claudin5 and VE-Cadherin, and blocking *miR-126* activity in HLECs phenocopies these

11 changes in expression. Importantly, overexpression of *miR-126* in GATA2$^{\Delta HLEC}$ significantly

12 rescues the cell junction defects. Thus, our work defines a new mechanism of GATA2 and

13 uncovers *miR-126* as a novel regulator of mammalian lymphatic vascular development.

14

15

16

17 **Key words:** Lymphatic vasculature, lymphovenous valves, GATA2, miR-126, Claudin5, VE-

18 Cadherin.

19

20

2

1   **Non-standard abbreviations**

2   **LECs**, lymphatic endothelial cells; **LVs**, lymphatic valves; **LV-ECs**, lymphatic valve-forming

3   endothelial cells; **LVVs**, lymphovenous valves; **LVV-ECs**, lymphovenous valve-forming

4   endothelial cells; **HLEC**, primary human LECs; **OSS**, Oscillatory shear stress; **IHC**,

5   immunohistochemistry.

6

1 **INTRODUCTION**

2 The lymphatic vasculature is a hierarchically organized tissue that absorbs and returns

3 extravasated plasma fluids and digested lipids to the blood circulation (Tammela and Alitalo

4 2010; Chen et al. 2014). This fluid, commonly known as lymph, is absorbed by lymphatic

5 capillaries and transported via collecting lymphatic vessels. Lymphatic valves (LVs) within the

6 lymphatic vessels regulate the unidirectional flow of lymph. Finally, lymph is returned to the

7 blood circulation at the junction of jugular and subclavian veins through four lymphovenous

8 valves (LVVs).

9   Mutations in multiple genes are associated with lymphedema, a debilitating disease

10 characterized by the swelling of tissues, most obviously the limbs (Brouillard et al. 2014). Other

11 lymphatic anomalies include chylous ascites (fluid in the peritoneal cavity), chylothorax (fluid

12 around the lungs) or lymph reflex. These lymphatic vascular dysfunctions could arise from

13 anatomical defects in the vessels or valves, though in most cases the precise cause is unclear.

14   Heterozygous mutations in the zinc finger transcription factor *GATA2* are associated

15 with an array of hematopoietic disorders and lymphedema (Spinner et al. 2014). The

16 overlapping phenotypes of this disease include immune deficiency, myelodysplasia (MDS),

17 acute myeloid leukemia (AML), predisposition to mycobacterial infections and warts, hearing

18 loss and lymphedema (Spinner et al. 2014; Crispino and Horwitz 2017). Emberger syndrome,

19 caused by mutations in *GATA2*, is classified as deafness and primary lymphedema with

20 MDS/AML (Emberger et al. 1979; Ostergaard et al. 2011; Kazenwadel et al. 2012).

21 Approximately 11-30% of people with mutated *GATA2* develop lymphedema (Ostergaard et al.

22 2011; Kazenwadel et al. 2012; Spinner et al. 2014; Donadieu et al. 2018). Donadieu *et al.*

23 noted that patients with *GATA2* mutations tend to develop lymphedema early, in the first

24 decade of life. In summary, early-onset lymphedema with incomplete penetrance is associated

25 with *GATA2*-heterozygous mutations. We need better insight into the molecular mechanisms

4

1 of GATA2 activity to understand the causes of lymphedema in Emberger syndrome patients.

2   Mouse models have revealed that GATA2 is critical for the development of a variety of

3 cell types including hematopoietic cells, neurons, pituitary glands, urinogenital system and the

4 endothelium (Zhou et al. 1998; Zhou et al. 2000; Craven et al. 2004; Charles et al. 2006;

5 Khandekar et al. 2007; Lim et al. 2012). *Gata2$^{-/-}$* mice die at embryonic day (E)10 just as

6 lymphatic endothelial cells (LECs) are starting to be specified. Conditional deletion of *Gata2*

7 from all endothelial cells during mouse development results in severely edematous embryos

8 with small blood-filled lymph sacs (Lim et al. 2012; Frye et al. 2018). Conditional deletion of

9 *Gata2* in LECs results in mispatterned dermal lymphatic vessels, and a loss of lymphatic

10 valves (LVs) (Kazenwadel et al. 2015; Frye et al. 2018). In addition, E12.5 or older embryos

11 with a conditional deletion of *GATA2* in all endothelial cells or LECs lack LVVs (Kazenwadel et

12 al. 2015; Geng et al. 2016; Frye et al. 2018). Thus, GATA2 is essential for proper development

13 of the lymphatic vasculature.

14   *In vitro* experiments have revealed several molecular functions of GATA2. A stiff extra

15 cellular matrix (ECM) triggers GATA2-dependent activation of *VEGFR2* expression in blood

16 endothelial cells (Mammoto et al. 2009). In contrast, a soft-ECM enhances *GATA2* expression

17 in primary human LECs (HLECs) and, in turn, induction of *VEGFR3* (Frye et al. 2018). This

18 mechanistic relationship has been proposed to be critical for LEC migration from the cardinal

19 vein and could explain the small lymph sacs in mice lacking *GATA2* in all endothelial cells.

20   Oscillatory shear stress (OSS), Wnt/β-catenin signaling and PROX1 are thought to be

21 the most-upstream regulators of LV and LVV formation, all of which activate *GATA2*

22 expression in HLECs (Kazenwadel et al. 2015; Sweet et al. 2015; Cha et al. 2016; Cha et al.

23 2018). OSS-induced GATA2 expression in HLECs is dependent on histone deacetylase 3

24 (HDAC3) (Janardhan et al. 2017). In turn, GATA2 is necessary for OSS-induced expression of

1   FOXC2 and Connexin 37 (Kazenwadel et al. 2015; Sweet et al. 2015). Furthermore, GATA2

2   directly associates with the regulatory elements of PROX1 in HLECs, and GATA2 knockdown

3   in HLECs downregulates the expression of PROX1 (Kazenwadel et al. 2015).

4   The current model built on these observations proposes that GATA2 regulates the

5   differentiation of valvular endothelial cells from progenitors by upregulating PROX1, FOXC2

6   and Connexin 37 in those cells. However, whether this model is accurate *in vivo* remains

7   unclear. Although LVV forming endothelial cells (LVV-ECs) differentiate at E12.0 with the

8   upregulation of PROX1, FOXC2, Connexin 37 and GATA2 in those cells (Geng et al. 2016),

9   whether GATA2 is necessary for LVV-EC differentiation is not known.

10

1   **RESULTS**

2   **GATA2 is necessary for the proper architecture of newly differentiated LVV-ECs**

3   Previous reports including ours have used pan-endothelial Cre lines for deleting *Gata2*

4   (Kazenwadel et al. 2015; Geng et al. 2016; Frye et al. 2018). *Gata2* has also been deleted in

5   the lymphatic vasculature in a mosaic manner using tamoxifen-inducible Cre lines

6   (Kazenwadel et al. 2015; Frye et al. 2018). Here, we used *Lyve1-Cre* (Pham et al. 2010) to

7   obtain uniform, constitutive deletion of *Gata2* (Charles et al. 2006) in the lymphatic

8   vasculature. As anticipated *Lyve1-Cre;Gata2$^{f/f}$* (*Gata2^{LECKO}*) embryos recapitulated the

9   previously reported lymphatic vascular phenotypes. Specifically, E16.5 *Gata2^{LECKO}* embryos

10 possessed blood-filled lymphatic vessels, which were dilated and had fewer branch points. The

11 mutant embryos also lacked LVs and LVVs (**Figure 1** and data not shown).

12   To investigate a potential role for GATA2 in LVV-EC differentiation, we used numerous

13 LVV-EC markers (PROX1^{high}, FOXC2^{high}, Connexin37 (GJA4), integrin-α5, integrin-α9) to

14 analyze E12.0 embryos (**Figure 2A, B** and **Figure 2- Figure Supplement 1A-H**). LVV-EC

15 numbers were not significantly different between control and *Gata2^{LECKO}* embryos littermates

16 at this stage (**Figure 2C**). We also analyzed E12.0 Tie2-Cre*;Gata2$^{f/f}$* embryos in which *Gata2*

17 is deleted from all endothelial cells at a much earlier time point (Kisanuki et al. 2001). E12.0

18 Tie2-Cre*;Gata2$^{f/f}$* embryos had LVV-ECs (**Figure 2- Figure Supplement 2**), excluding the

19 possibility that the LVV-ECs observed in *Gata2^{LECKO}* embryos is due to inefficient deletion of

20 *Gata2* by *Lyve1-Cre.* Thus, LVV-EC differentiation is normal in *Gata2^{LECKO}* mutants.

21   To examine LVV-ECs further, we used correlative fluorescent microscopy followed by

22 scanning electron microscopy (SEM) to visualize the developing LVV-ECs at high resolution

23 (Geng et al. 2016). First, we analyzed sagittal sections along the cardinal vein of E12.0

24 Tg(Prox1-tdTomato) embryos by confocal microscopy (Gong et al. 2003). We observed

25 tdTomato^{high} LVV-ECs in both control and *Gata2^{LECKO}* backgrounds (**Figure 2D, E**). SEM on

7

1 these same samples revealed individual LVV-ECs that are elongated and aligned

2 perpendicular to the direction of blood flow in control E12.0 embryos (**Figure 2D''**, pseudo

3 colored in green)**.** The rest of the venous endothelium was quiescent with cobblestone-like

4 appearance. In contrast, SEM revealed that the LVV-ECs in E12.0 Tg(Prox1-tdTomato);

5 *Gata2^{LECKO}* embryos are round and not aligned perpendicular to blood flow (**Figure 2- Figure**

6 **Supplement 3)**. In addition, LVV-ECs also appear dysplastic in E12.0 Tg(Prox1-tdTomato);

7 *Gata2^{LECKO}* embryos (**Figure 2E''**, pseudo colored in green, and **Figure 2- Figure**

8 **Supplement 3)**. Based on these observations we conclude that GATA2 is not necessary for

9 the differentiation of LVV-EC or for the upregulation of PROX1 or FOXC2 in those cells.

10 However, GATA2 is necessary for the proper architecture of the newly formed LVV-ECs.

11

12 **GATA2 is necessary for the maintenance and morphogenesis of LVV-ECs**

13 By E12.5, control embryos displayed LVV-ECs in the venous walls and two-well formed LVVs

14 (**Figure 3A,** arrows). In contrast, E12.5 *Gata2^{LECKO}* embryos had very few LVV-ECs in the

15 venous walls and lacked clearly defined LVVs (**Figure 3B,** arrow). In addition, E12.5 Tg(Prox1-

16 tdTomato) control embryos exhibited two tightly aggregated clusters of tdTomato^{high} LVV-ECs

17 (**Figure 3C**, arrows), and SEM of one these clusters revealed elongated LVV-ECs that formed

18 an opening in the middle (LVV) to permit lymph return to the blood circulation (**Figure 3C''**,

19 arrowhead). In contrast, Tg(Prox1-tdTomato);*Gata2^{LECKO}* embryos lacked tdTomato^{high} LVV-

20 ECs (**Figure 3D**), and SEM of the LVV-forming region revealed a smooth luminal surface,

21 devoid of LVVs (**Figure 3D''**).

22   Fluorescent reporter proteins such as tdTomato have a long half-life and can remain

23 within cells for several days after the reporter gene is shut off (Muzumdar et al. 2007).

24 However, tdTomato^{high} LVV-ECs in Tg(Prox1-tdTomato);*Gata2^{LECKO}* embryos disappear within

25 12 hrs (between E12.0 to E12.5), suggesting that LVV-ECs were eliminated either by cell

1   death or by detachment and removal via the bloodstream. To verify the loss of LVV-ECs, we

2   performed lineage tracing. We generated *Prox1-CreERT2;R26$^{+/tdTomato}$* embryos in a control

3   (wild-type) and *Gata2$^{f/f}$* background, treated pregnant dams with tamoxifen at E10.5, and

4   evaluated embryos at E15.5. *R26$^{+/tdTomato}$* allowed us to lineage trace the PROX1$^+$ cells (LECs

5   and LVV-ECs). Whereas entire LVVs were tdTomato$^+$ in control embryos (**Figure 3E,** arrows),

6   LVVs were absent in *Prox1-CreERT2;Gata2$^{f/f}$;R26$^{+/tdTomato}$* embryos (**Figure 3F**). Importantly,

7   the LVV-forming area of *Prox1-CreERT2;Gata2$^{f/f}$;R26$^{+/tdTomato}$* embryos had very few labeled

8   cells (**Figure 3F**), consistent with a loss of LVV-ECs in embryos lacking *Gata2*.

9   LVV-ECs in E12.0 or E12.5 control embryos did not express the proliferation marker

10   phospho-histone 3 (PHH3) (data not shown), indicating that these cells do not proliferate.

11   Therefore, the lack of LVV-ECs in *Gata2^{LECKO}* embryos does not reflect impaired proliferation.

12   We observed a few activated Casp3$^+$ apoptotic cells within the lymph sacs of *Gata2^{LECKO}*

13   embryos, but LVV-ECs did not appear to be labeled by this marker for apoptosis (data not

14   shown). Together these results indicate that GATA2 is not required for the differentiation of

15   LVV-ECs or for their survival or proliferation. We however cannot exclude the possibility of cell

16   death with the simultaneous detachment of LVV-ECs into the bloodstream.

17   In summary, GATA2 regulates the morphology of LVV-EC, maintains their presence in

18   the valve-forming region, and regulates their morphogenesis into LVVs.

19

20   **GATA2 is not necessary for the upregulation of PROX1 and FOXC2 in VVs, LVs or AoVs**

21   GATA2 is also expressed in LVs, venous valves (VVs) and aortic valves (AoVs) (Kazenwadel

22   et al and data not shown) (Kazenwadel et al. 2015). Hence, we tested whether GATA2 is

23   necessary for the differentiation of those valvular endothelial cells. VVs of the jugular vein exist

24   close to LVVs at E16.5. VV-forming endothelial cells (VV-ECs) differentiate at this region at

25   around E14.5. Control E15.5 embryos displayed LVVs (**Figure 4A**, arrows) and developing

1   VVs invaginating into the veins (**Figure 4A**, arrowheads). In contrast, *Gata2*^{LECKO} E15.5

2   embryos lacked LVVs, and PROX1^{high} VV-ECs were not invaginating into the veins (**Figure**

3   **4B**, arrowheads). Expression of FOXC2 is also unaffected in the VV-ECs of *Gata2*^{LECKO}

4   embryos (data not shown). Thus, GATA2 is not necessary for the differentiation of VV-ECs.

5   We deleted *Gata2* using *Prox1*$^{+/Cre}$ to remove GATA2 from the PROX1^{+} aortic valve

6   endothelial cells (AoV-ECs) (Srinivasan et al. 2010). PROX1 and *Prox1*$^{+/Cre}$ are expressed in

7   AoV-ECs as early as E12.5 (data not shown). As shown in **Figure 4C, D,** expression of

8   PROX1 and FOXC2 was unaffected in the AoV-ECs of E14.5 *Prox1*$^{+/Cre}$*;Gata2*$^{f/f}$ embryos. We

9   also did not observe any obvious differences in the expression of PROX1 and FOXC2 in E16.5

10   *Prox1*$^{+/Cre}$*;Gata2*$^{f/f}$ embryos (data not shown). Thus, GATA2 is not required to upregulate

11   PROX1 and FOXC2 in AoV-ECs or to maintain these cells.

12   We noticed that in contrast to *Gata2*^{LECKO} embryos *Prox1-CreERT2;Gata2*$^{f/f}$ embryos do

13   not develop blood-filled lymphatic vessels even though they lack LVVs (**Figure 3F**). Blood-

14   filled lymphatic vessels could affect LV development (Sweet et al. 2015). Therefore, we

15   analyzed *Prox1-CreERT2;Gata2*$^{f/f}$ embryos to investigate the role of GATA2 in LV

16   development. We exposed pregnant dams carrying *Prox1-CreERT2;Gata2*$^{f/f}$ embryos to

17   tamoxifen at E14.5 and harvested the embryos at E16.5. Analysis of the skin and mesenteric

18   lymphatic vessels revealed that LV-EC clusters were present in both control and mutant

19   embryos, although those numbers were reduced in the mutants (**Figure 4E-J**). In contrast,

20   E18.5 *Prox1-CreERT2;Gata2*$^{f/f}$ embryos that were exposed to tamoxifen at E14.5 completely

21   lacked LVs (data not shown). Hence, these results suggest that GATA2 is not necessary for

22   the differentiation of LV-ECs, but it is necessary to maintain those cells.

23   Together, these results indicate that GATA2 is not necessary for the differentiation of

24   LVV-ECs, VV-ECs, LV-ECs or AoV-ECs or for the upregulation of PROX1 and FOXC2 in those

25   cells. However, GATA2 is necessary to maintain vascular valve endothelial cells (LVV-ECs

1 and VV-ECs) and promote their morphogenesis.

2

3 *EGFL7* and *ANGPT2* are regulated by GATA2 in HLECs

4 To identify potential genes regulated by GATA2 *in vivo*, we examined GATA2-dependent gene

5 expression in HLECs. We treated HLECs with lentiviral particles expressing shGFP or

6 shGATA2, harvested them 72 hrs later and performed RNA-seq. We performed this

7 experiment in triplicate, and Principle Component Analysis confirmed the consistency in gene

8 expression changes among the triplicates (**Figure 5A**). We pursued genes whose expression

9 is significantly (p<0.05) different between shGFP- and shGATA2-treated HLECs, with Log_2 fold

10 change (FC) >0.5 or Log_2 FC<-0.5. According to these criteria, 1009 genes were significantly

11 downregulated and 617 genes were significantly upregulated upon depletion of *GATA2* in

12 HLECs (**Figure 5B** and **Supplementary File 1**). *GATA2* was dramatically downregulated (Log_2

13 FC = -2.08) in shGATA2-treated HLECs, as expected (**Supplementary File 1**). We did not

14 observe significant changes in the expression of *PROX1, FOXC2 or FLT4*. However, a number

15 of other genes that regulate vascular development were differentially expressed in shGATA2-

16 treated HLECs (**Figure 5C**).

17 Among the top 50 most down regulated genes only *GATA2, Angiopoietin 2* (*ANGPT2*)

18 and *EGF-like domain-containing protein 7 (EGFL7)* are reported to be necessary for embryonic

19 survival and vascular development (http://www.informatics.jax.org). *EGFL7* (NM_201446) is a

20 secreted protein that was first reported as a regulator of vascular lumen formation (Parker et

21 al. 2004). EGFL7 also regulates blood endothelial cell migration, contractility and adhesion

22 (Charpentier et al. 2013). *EGFL7* is also the host gene for miR-126, which is the first

23 endothelial cell-specific microRNA to be reported (Lagos-Quintana et al. 2002). Interestingly,

24 EGFL7 is not required for the survival of mice as long as miR-126 is intact (Kuhnert et al.

25 2008). In contrast, most *miR-126*^{-/-} embryos die in utero with severe edema (Kuhnert et al.

11

1   2008; Wang et al. 2008). Importantly, GATA2 was recently reported to regulate EGFL7 and

2   miR-126 in blood vascular endothelial cells (Hartmann et al. 2016). However, the lymphatic

3   vasculature specific roles of miR-126 remain unknown.

4   ANGPT2 is a secreted molecule and a ligand of TIE2 (also known as TEK). In blood

5   endothelial cells, ANGPT2 is an antagonist of TIE2. The TIE2/ANGPT2 interaction in blood

6   endothelial cells results in VE-PTP-mediated downregulation of VE-Cadherin (Souma et al.

7   2018). In contrast, in LECs ANGPT2 is an agonist of TIE2 due to the absence of VE-PTP.

8   Deletion of *Angpt2* results in a strain-specific postnatal lethality in mice due to severe

9   chylothorax (Gale et al. 2002; Dellinger et al. 2008). $Angpt2^{-/-}$ mice lack LVs and have

10   defective cell junctions. Whether ANGPT2 is necessary for LVV development is unknown.

11   To validate whether ANGPT2 and EGFL7 are GATA2 targets, we used CRISPR/Cas9

12   to generate *GATA2* knockout in a distinct HLEC cell line (HLEC-2). Western blotting and DNA

13   sequencing confirmed the deletion of *GATA2* in GATA2^{ΔHLEC} (**Figure 5D** and **Figure 5- Figure**

14   **Supplement 1**). Importantly, the protein levels of both EGFL7 and ANGPT2 were reduced in

15   GATA2^{ΔHLEC} cells compared to controls, whereas PROX1 was unaffected (**Figure 5D**). In

16   addition, after extracting miRNA's from the cells we determined by qRT-PCR that miR-126 is

17   significantly downregulated in GATA2^{ΔHLEC} cells compared to controls (**Figure 5D**).

18   In summary, *EGFL7,* miR-126 and *ANGPT2* are consistently downregulated by the

19   knockdown or knockout of GATA2 in HLECs.

20

21   ***MiR-126* is a physiologically important target of GATA2 in the lymphatic vasculature**

22   To investigate the physiological relevance of these candidate GATA2 target genes, we

23   compared their expression in the LVV-ECs of E12.0 control and *Gata2^{LECKO}* embryos.

24   ANGPT2 was not expressed in LVV-ECs at E12.0 although it appears at E14.5 (**Figure 6**).

1   *Angpt2*$^{-/-}$ embryos lacked LVs and had defective lymphatic vessel patterning as reported

2   previously (data not shown) (Gale et al. 2002; Dellinger et al. 2008). However, *Angpt2*$^{-/-}$

3   embryos retained normal looking LVVs and VVs (**Figure 6**). These observations suggested

4   that GATA2-dependent regulation of *Angpt2* is not involved in LVV and VV development.

5   Hence, we focused our attention on miR-126 for the rest of this work.

6   *In situ* hybridization revealed high expression of *miR-126* in LVV-ECs of E12.0 control

7   embryos but not *Gata2*^{LECKO} embryos (**Figure 7A, B,** arrows). Similarly, E18.5 control embryos

8   expressed EGFL7 in the LECs of mesenteric lymphatic vessels and in LVs (**Figure 7C,** arrow),

9   whereas EGFL7 expression was dramatically reduced in the LECs of E18.5 *Prox1-*

10   *CreERT2*;*Gata2*$^{f/f}$ embryos in which *Gata2* deletion was induced by tamoxifen injection at

11   E14.5 (**Figure 7D**). As mentioned previously, the mutants lacked LVs. Thus, GATA2 is

12   required for *EGFL7/miR-126* expression in the developing lymphatic vasculature.

13   GATA2 associates with the promoter of *EGFL7/miR-126* in human umbilical vein

14   endothelial cells (HUVECs) (Hartmann et al. 2016). We performed chromatin

15   immunoprecipitation (ChIP) using an anti-GATA2 antibody and determined that GATA2

16   associates with this promoter region in HLECs as well (**Figure 7E**). These results suggest that

17   *EGFL7/miR-126* is a direct target of GATA2 in the lymphatic vasculature.

18   As mentioned previously *Egfl7*$^{-/-}$ mice that retain miR-126 are phenotypically normal

19   (Kuhnert et al. 2008). Therefore, we analyzed *miR-126*$^{-/-}$ embryos, which display severe edema

20   (Wang et al. 2008). LVVs and VVs were absent in the jugulo-subclavian vein junction of E16.5

21   *miR-126*$^{-/-}$ embryos compared to wild type (**Figure 8A, B**). LVV-ECs were present in E12.0

22   *miR-126*$^{-/-}$ embryos (**Figure 8C, D**), indicating that *miR-126* is not necessary for the

23   differentiation of LVV-ECs, but for their maintenance. *MiR-126*$^{-/-}$ embryos had dilated

24   mesenteric lymphatic vessels that lack LVs (**Figure 8E, F**), and the lymphatic vessels in the

25   dorsal skin are hypoplastic (**Figure 8G, H**).

1   Overall, these observations demonstrate that GATA2 regulates miR-126 both in vitro

2   and in vivo. Furthermore, a significant level of phenotypic similarity between *Gata2*[LECKO] and

3   *miR-126*^{-/-} embryos, including defects in the maintenance of LVVs and lymphatic vessel

4   patterning indicate that miR-126 is likely a physiologically relevant target of GATA2 in the

5   lymphatic vasculature.

6

7   **GATA2 regulates lymphatic endothelial cell junctions via *miR-126***

8   The Ras MAPK pathway inhibitor *Sprouty related, EVH1 domain-containing protein 1 (Spred1)*

9   is a target of miR-126 in blood endothelial cells (Fish et al. 2008; Wang et al. 2008), and

10   deletion of *Spred1* rescues the blood vascular defects of *miR-126*^{-/-} embryos (Wang et al.

11   2008; Zhou et al. 2016). To investigate a potential role for *Spred1* in the lymphatic vasculature,

12   we examined *Spred1* knockout embryos. *miR-126*^{-/-} embryos displayed edema as anticipated

13   **(Figure 9A, B)**. *Spred1*^{-/-} embryos did not have edema or any obvious defects in LVVs or

14   lymphatic vessels **(Figure 9C-H)**. Importantly, deletion of *Spred1* did not rescue the lymphatic

15   vascular defects of *miR-126*^{-/-} embryos **(Figure 9C-H)**. Thus, miR-126 regulates lymphatic

16   vascular development independently of *Spred1*.

17   To identify the relevant targets of miR-126 we performed RNA-seq in HLECs expressing

18   an "miR-126 sponge" (Gentner et al. 2009; Lechman et al. 2012) to sequester *miR-126* from its

19   endogenous targets (**Supplementary File 1**). Using the same criteria described above we

20   identified 1058 genes that were upregulated and 873 genes that were downregulated by miR-

21   126 sponge. SPRED1 and PIK3R2, which is another reported target of miR-126 in blood

22   endothelial cells, were not in the list (Fish et al. 2008). *PROX1, FOXC2, FLT4* and *GATA2*

23   were also not found in this list. By comparing these genes with the GATA2 regulated genes we

24   identified 125 shared downregulated genes and 72 shared upregulated genes (data not

14

1   shown). DAVID gene annotation was used to classify the shared genes (Huang da et al.

2   2009a; Huang da et al. 2009b). Sixteen clusters were observed among the downregulated

3   genes with 42 membrane-associated proteins constituting the largest group. Nine clusters

4   were observed among upregulated genes, which included the keywords Membrane,

5   Cytoskeleton, Microtubule, Metalloprotease, Rap1 signaling and Cell junctions. All of the terms

6   identified among upregulated and downregulated genes are relevant to the regulation of

7   vascular integrity (Dudek and Garcia 2001; Chrzanowska-Wodnicka 2017). Therefore, we

8   analyzed the expression of cell junction molecules in $Gata2^{LECKO}$ and $miR\text{-}126^{-/-}$ embryos.

9   Claudin5 expression was dramatically downregulated in the lymphatic vessels of E16.5 $miR\text{-}$

10    $126^{-/-}$ embryos (**Figure 10A, A', B, B''**). Additionally, whereas VE-Cadherin was uniformly

11   expressed along the cell junctions of control embryos, it displayed discontinuous expression in

12    $miR\text{-}126^{-/-}$ embryos (**Figure 10A'', A''', B'', B'''**). We identified identical defects in VE-

13   Cadherin and Claudin5 expression in E16.5 $Gata2^{LECKO}$ embryos (**Figure 10C, D**). In addition,

14   VE-Cadherin expression was disorganized in the mesenteric lymphatic vessels of E18.5

15    $Prox1\text{-}CreERT2; Gata2^{f/f}$ embryos that were exposed to tamoxifen at E14.5 (**Figure 10E, F**).

16   Thin sections of LVV-ECs from E12.0 control and $Gata2^{LECKO}$ embryos did not reveal

17   any obvious differences in VE-Cadherin or Claudin5 expression (**Figure 2- Figure**

18   **Supplement 1M-P**). However, LVV-ECs delaminate from the walls of veins at E12.0 before

19   reassembling in multiple layers to form mature LVVs at E12.5 (Geng et al. 2016). Such a rapid

20   morphogenesis of LVV-ECs is likely to involve dramatic reorganization of cell junctions. And,

21   the deletion of VE-Cadherin from the lymphatic vasculature was recently reported to prevent

22   the formation of LVs (Hagerling et al. 2018). Hence, we are tempted to speculate that a defect

23   in the reorganization of cell junctions might be the cause of LVV-EC disappearance in E12.5

24    $Gata2^{LECKO}$ embryos.

15

1   Given that the lymphatic vessels of E16.5 *Gata2*^{LECKO} and *miR-126*$^{-/-}$ embryos had

2   defective cell junctions, we examined Claudin5 and VE-Cadherin expression in HLECs.

3   Claudin5 and VE-Cadherin were uniformly expressed around the entire periphery in ~80% of

4   control HLECs (**Figure 11A, B**). In contrast, the intensity of Claudin5 expression was

5   dramatically reduced in GATA2^{ΔHLEC} cells (**Figure 11C**). In addition, the localization of VE-

6   Cadherin was defective in GATA2^{ΔHLEC} cells with numerous gaps (**Figure 11D,** arrowheads).

7   To determine whether miR-126 also influences Claudin5 expression in HLECs, we

8   overexpressed the "miR-126 sponge" (Lechman et al. 2012) in HLECs, and observed a

9   significant reduction in Claudin5 expression and defective VE-Cadherin localization (**Figure**

10   **11E-H**). Thus, both GATA2 and *miR-126* are regulators of Claudin5 expression and VE-

11   Cadherin localization *in vitro* and *in vivo*.

12   To determine whether GATA2 regulates Claudin5 via miR-126, we overexpressed *miR-*

13   *126* in GATA2^{ΔHLEC} cells using lentiviral particles (Amendola et al. 2009). We observed a

14   partial, yet significant rescue of Claudin5 expression in GATA2^{ΔHLEC} expressing *miR-126*. In

15   addition, miR-126 significantly rescued the localization of VE-Cadherin at the cell junctions

16   (**Figure 11I-G**). These results suggest that GATA2 regulates adherens and tight junctions in

17   LECs through miR-126.

18

1   **DISCUSSION**

2   In this work, we have discovered that although GATA2 is not necessary for LVV-EC

3   differentiation, it is required for their maintenance. Further, GATA2 is important for LVV-ECs

4   and LECs to align appropriately with respect to the direction of fluid flow. GATA2 activates the

5   expression of *miR-126* in LVV-ECs and LECs. The lymphatic vascular defects of mice lacking

6   GATA2 or *miR-126* are strikingly similar, and both GATA2 and *miR-126* are necessary for the

7   expression of cell junction molecules Claudin5 and VE-Cadherin. Importantly, *miR-126* could

8   significantly rescue cell junction defects in HLECs lacking GATA2. Based on our results we

9   propose a model in which GATA2 regulates LVV morphogenesis and lymphatic vascular

10   maturation by maintaining proper cell junctions via *miR-126* (**Figure 12**).

11   Valves normally develop at locations of disturbed flow. Hence, pioneering work by

12   Sabine *et al.* proposed OSS as the most upstream regulator of valve development (Sabine et

13   al. 2012). Significant advances have been made since this initial report. We showed that

14   Wnt/ß-catenin signaling enhances the expression of FOXC2 and GATA2 in response to OSS

15   (Cha et al. 2016), and that PROX1 is necessary for the activity of Wnt/ß-catenin signaling (Cha

16   et al. 2018). Elegant studies have shown that GATA2 enhances the expression of FOXC2 in

17   an OSS-dependent manner (Kazenwadel et al. 2015; Sweet et al. 2015). Despite this

18   knowledge, the precise role of OSS in valve development is unknown. Cytoskeleton, cell-cell

19   and cell-matrix interactions are critical regulators of mechanotransduction (Ingber 2006; Hahn

20   and Schwartz 2009). Therefore, our finding that GATA2 regulates VE-Cadherin and Claudin5

21   expression through miR-126 provides a mechanistic explanation for GATA2-mediated

22   mechanotransduction. Surprisingly, GATA2 is not necessary for the upregulation of FOXC2

23   expression in LVV-ECs *in vivo*. Therefore, we suggest that the OSS/GATA2/FOXC2 axis is not

24   necessary for the differentiation of valvular endothelial cells. Instead, OSS might be important

25   for lymphatic vessel patterning and the maintenance of FOXC2 expression in valvular

17

1   endothelial cells. In line with this model, the mechanosensory ion channel PIEZO1 was

2   recently shown to be necessary for LV development (Nonomura et al. 2018; Choi et al. 2019).

3   Just like GATA2, PIEZO1 is not necessary for the differentiation of LV-ECs (Nonomura et al.

4   2018). Instead, PIEZO1 maintains LV-ECs and regulates their morphogenesis into LVs.

5   　　　Several interesting questions arise from our work for future exploration. Recently,

6   Kontarakis et al reported that they do not observe any obvious edema or lymphatic defects in

7   *miR-126$^{-/-}$* embryos (Kontarakis et al. 2018). However, we consistently observe severe edema

8   and lymphatic vascular defects in *miR-126$^{-/-}$* embryos (**Figure 9B)**. The reasons for these

9   phenotypic differences remain to be determined. Neither GATA2 nor *miR-126* regulates

10   Claudin5 at the mRNA level (**Supplementary File 1** and data not shown). Hence, how *miR-*

11   *126* regulates Claudin5 is currently unknown. The mechanisms behind the abnormal patterning

12   of VE-Cadherin are also not known. It will be of interest to generate mice lacking cell junction

13   molecules VE-Cadherin and Claudin5, to determine if they recapitulate any of the phenotypes

14   of *Gata2^{LECKO}* and *miR-126$^{-/-}$* embryos. Future work should also address whether modulators

15   of cell-ECM interaction and cadherin stability such as ADAM19 and MMP15, which are

16   significantly upregulated in shGATA2- and miR-126 sponge-treated HLECs, play

17   physiologically important roles in the development of the lymphatic vasculature.

18   　　　How GATA2 regulates blood-lymph separation is not yet clear. LVV-ECs do not form

19   until E12.0 (Srinivasan and Oliver 2011; Geng et al. 2016). However, the lymph sacs of E11.5

20   *Gata2^{LECKO}* embryos are blood filled (data not shown). Furthermore, as mentioned previously,

21   *Prox1-CreERT2;Gata2$^{f/f}$* embryos did not have blood-filled lymphatics phenotype despite the

22   absence of LVVs (**Figure 3E, F**). These observations suggest that GATA2 is regulating blood-

23   lymph separation through an LVV-independent mechanism.

24   　　　Platelet-expressed CLEC2 and LEC-expressed podoplanin play a critical role in blood-

25   lymph separation (Fu et al. 2008; Bertozzi et al. 2010). Expression of podoplanin is not

18

1 affected in *Gata2^LECKO* embryos, and platelets are present in *Gata2^LECKO* embryos (data not

2 shown). Together these results suggest that GATA2 regulates blood-lymph separation

3 independent of the platelet/LEC interaction. As GATA2 regulates the expression of numerous

4 genes in blood endothelial cells, and as LECs originate predominantly from embryonic veins,

5 we are tempted to speculate that GATA2 might be regulating blood-lymphatic separation by

6 maintaining blood vascular integrity during the migration of LECs from the veins.

7 Only some patients with mutations in GATA2 will develop lymphedema. A subset of

8 mice lacking one allele *Gata2* in endothelial cells display a dilated thoracic duct with reduced

9 lymph flow (Kazenwadel et al. 2015), but they have a normal lifespan and do not develop any

10 obvious symptoms of the human disease such as leukemia, bacterial infections or warts (our

11 unpublished observations). Compared to humans, mice experience less severe gravitational

12 load in the lymphatic vessels of their limbs (Castorena-Gonzalez et al. 2018), which might

13 underlie the absence of obvious lymphatic defects in *Gata2^{+/-}* mice. Alternative explanations

14 are also possible. Secondary mutations in *ASXL1* are frequently observed in Emberger

15 syndrome patients who develop MDS/AML (Crispino and Horwitz 2017). Similarly, a "second

16 hit" in the targets of GATA2, such as miR-126, might be required to trigger the onset of

17 lymphedema in human patients. Non-coding RNAs are powerful biomarkers of human disease

18 due to their ability to be detected in bodily fluids (Van Roosbroeck et al. 2013). Whether

19 circulating miR-126 levels might predict the onset of lymphedema in Emberger syndrome

20 patients needs to be determined.

21 Finally, several miRNA mimics and miRNA inhibitors have entered Phase I, Phase II

22 and preclinical trials and approaches to deliver them are rapidly improving (Rupaimoole and

23 Slack 2017). Hence, we are excited about the possibility that miR-126 might one day be used

24 as a "drug" to treat lymphedema in Emberger syndrome patients.

25

1 **MATERIALS AND METHODS**

2 **Antibodies**

3 Primary antibodies for immunohistochemistry: rabbit anti- PROX1 (Cat# 11-002, Angiobio, San

4 Diego, CA, USA), goat anti-human PROX1 (Cat# AF2727, R&D Systems, Minneapolis, MN,

5 USA), sheep anti-mouse FOXC2 (Cat# AF6989, R&D Systems, Minneapolis, MN, USA), goat

6 anti-mouse VEGRF3 (Cat# AF743, R&D Systems, Minneapolis, MN, USA), rat anti-mouse

7 CD31 (Cat# 553370, BD Pharmingen, San Jose, CA, USA), goat anti-mouse ITGA9 (Cat #

8 AF3827, R&D Systems, Minneapolis, MN, USA), rat anti-mouse VE-Cadherin (Cat# 550548,

9 BD Pharmingen, San Jose, CA, USA), hamster anti-mouse PDPN (Cat# 127401, Biolegend,

10 San Diego, CA, USA), rat anti-mouse ITGA5 (Cat #553319, BD Pharmingen, San Jose, CA,

11 USA), goat anti-mouse GATA2 (Cat #AF2046, R&D Systems, Minneapolis, MN, USA), rabbit

12 anti-mouse CX37 (Cat #40-4200, Life technologies, Grand Island, NY, USA), rabbit anti-mouse

13 LAMA5 (Abcam, Cambridge, MA, USA), rabbit anti-human Fibronectin (Cat# ab2413, Abcam,

14 Cambridge, MA, USA), Goat anti-human ANGPT2 (Cat# AF623, R&D Systems, Minneapolis,

15 MN, USA), goat anti-mouse EGFL7 (Cat # AF3089, R&D Systems, Minneapolis, MN, USA),

16 rabbit anti-mouse CLDN5 (Cat #34-1600, Thermo Fisher Scientific, Rockford, IL, USA), rabbit

17 anti-mouse LYVE-1 (Cat # 11-034, Angiobio, San Diego, CA, USA).

18

19 Secondary antibodies for immunohistochemistry: Cy3-conjugated donkey anti-rabbit, Cy3-

20 conjugated donkey anti-sheep, and Cy5-conjugated donkey anti-rat antibodies were purchased

21 from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Alexa 488-conjugated

22 donkey anti-goat, Alexa 488-conjugated goat anti-chicken, and Alexa 488-conjugated donkey

23 anti-rat were purchased from Life Technologies (Grand Island, NY, USA).

24

1 Primary antibodies for Western blotting: mouse anti- β-Actin (Cat# A5441, Sigma-Aldrich, St.

2 Louis, MO, USA), goat anti-human PROX1 (Cat# AF2727, R&D Systems, Minneapolis, MN,

3 USA), goat anti-mouse GATA2 (Cat #AF2046, R&D Systems, Minneapolis, MN, USA), goat

4 anti-mouse EGFL7 (Cat # AF3089, R&D Systems, Minneapolis, MN, USA) and mouse anti-

5 human ANGPT2 (Cat# DY623, R&D Systems, Minneapolis, MN, USA), Rabbit anti-human

6 GAPDH (Cat# PAB13195, Abnova, Taipei City, Taiwan, R.O.C).

7

8 HRP-conjugated secondary antibodies for Western blotting: goat anti-mouse IgG, goat anti-

9 rabbit IgG, donkey anti-goat IgG, and donkey anti-sheep IgG from Santa Cruz Biotechnology.

10

11 **Cells**

12 We used de-identified primary human lymphatic endothelial cells (HLECs) for experiments.

13 HLEC-1 were from Lonza (CC-2812) and were used for RNA-seq analysis with shGATA2.

14 HLEC-2 (Lonza, CC-2516) were used for all other experiments. HLECs were grown on

15 firbonectin-coated plates or glass slide and were maintained in EBM2 media from Lonza. All

16 experiments were conducted using passage 5-6 cells. HLECs were treated as potential

17 biohazards and were handled according to institutional biosafety regulations.

18

19 **Chromatin Immunoprecipitation**

20 ChIP assays were performed using EZ-ChIP kit (MilliporeSigma, Burlington, MA, USA)

21 according to the manufacturer's instructions. Around 1.0 X 10^7 HLECs were used per ChIP.

22 Briefly, HLECs were grown on culture dish at around 100% confluence. Subsequently, HLECs

23 were fixed in 1% formaldehyde for 10 min at room temperature and glycine at a final

24 concentration of 0.125 M was added for 5 min. Cells were washed with 20 mL of ice cold PBS

21

1   twice (ten minutes each) and harvested. Cells were lysed and sonicated as described

2   previously described (Cha et al. 2016; Cha et al. 2018).

3   Chromatin immunoprecipitation was performed using 3.0 μg of goat anti-mouse GATA2

4   (R&D Systems, Minneapolis, MN, USA) or 1.0 μg of normal goat IgG antibody (R&D Systems,

5   Minneapolis, MN, USA). Q-PCR following ChIP was performed using primers flanking the

6   predicted GATA2 binding sites or control sites of miR-126 promoter.

7

8   **Immunohistochemistry of tissues**

9   Immunohistochemistry on sections was done according to our previously published protocols

10   (Cha et al. 2016; Geng et al. 2016; Cha et al. 2018). Briefly, freshly collected embryos were

11   washed in 1X PBS and fixed in 4% paraformaldehyde (PFA) overnight at 4°C. Subsequently,

12   the embryos were washed 3X (10 minutes each) in cold PBS, incubated in 15% sucrose

13   overnight at 4°C and then in 30% sucrose at 4°C until fully submerged in the solution. Embryos

14   were then cryo embedded in OCT solution (Sakura, Tokyo, Japan). 12 μm thick cryosections

15   were prepared using a cryotome (Thermo Fisher Scientific, Model: HM525 NX) and

16   immunohistochemistry was performed using the indicated antibodies. E11.5 embryos were

17   sectioned in a transverse orientation and E12.0-E16.5 embryos were sectioned frontally.

18   Several consecutive sections were analyzed to determine the presence or absence of LVVs

19   and VVs.

20   Whole mount immunohistochemistry using embryonic skin or guts was performed

21   according to our previous protocol (Cha et al. 2016; Cha et al. 2018). Either whole embryos or

22   isolated guts were washed in 1X PBS and fixed in 1% PFA for 1 hr to overnight (depending on

23   the antibody) at 4°C. Subsequently, the dorsal skins were isolated, washed and samples were

24   immunostained using the iDISCO protocol (Renier et al. 2014). Samples were visualized and

25   analyzed as described previously (Cha et al. 2016; Cha et al. 2018).

Immunostaining of cells

Cells were fixed in 1% PFA at room temperature for 30 min. Cells were subsequently permeabilized with 0.3 % triton X-100 for 10 min at room temperature, then washed with PBST (PBS + 0.1% Triton-X100) and blocked in 0.5% BSA PBST for 1hour at room temperature. Samples were incubated with primary antibodies at 4°C overnight. Samples were then washed with PBST and incubated with secondary antibodies for 2 hours at room temperature, and then washed with PBST three times (10 min each), mounted and visualized as previously described (Cha et al. 2016; Cha et al. 2018).

In situ hybridization

We used a kit to detect mmu-miR-126-3p by in situ hybridization (catalog number 339111, Qiagen, Germantown, MD, USA). Briefly, we fixed the embryos in 4% PFA overnight at 4°C. They were then soaked in sucrose, embedded in OCT and sectioned as described above. The sections were fixed in 4% PFA for 10 min at RT and washed in PBS. Subsequent steps were performed according to manufacturer's instructions.

Knockdown of GATA2

shGATA2 (TTAACAGGCCACTGACCATGAAGAAGGAA) was cloned into a pLV plasmid. Cyagen Bioscience (Santa Clara, CA, USA) generated the lentiviral particles using LentiPAC 293 cells. HLECs were seeded at 50-60% confluence on fribronectin-coated plates. The following day cells are infected with equal amounts of shcontrol or shGATA2 virus according to manufacturer protocol for 4-6 hrs in Opti-MEM medium and then changed to regular EBM2 media. After 2-3 days cells were harvested with Trizol (Invitrogen, Carlsbad, CA, USA) for RNA-seq study.

Knockout of GATA2 using CRISPR/Cas9

sgRNA1 (GGTCTGGGTGCAGACGGCAA), sgRNA2 (ATGCCAACCCCGCTCACGCG) and Cas9 were cloned into a pLV plasmid with puromycin selection marker. The translational start site ATG of *GATA2* is located between the recognition sites of sgRNA1 and sgRNA2. Cyagen Bioscience (Santa Clara, CA, USA) generated the lentiviral particles using LentiPAC 293 cells. HLEC-2 were seeded at 50-60% confluence on fibronectin-coated plates. The following day cells were infected with equal amount of control or GATA2 CRIPSPR/cas9 recombinant lentiviral particles according to manufacturer protocol for 4-6 hrs in Opti-MEM medium and then changed to regular EBM2 media. After 24 hrs cells were treated with 0.5µg/mL puromycin to select the cells. After 3 days 0.5µg/mL puromycin treatment almost all non-infected HLECs were dead. We used 5 days of 0.5µg/mL puromycin treatment for selecting GATA2$^{\Delta HLEC}$.

A gene-specific primer pair was used that could cover both sgRNA1 and sgRNA2 sequences, generating 330 bp long amplicons. The resulting PCR amplicons were purified using MinElute PCR purification kit (Qiagen). Sequencing library was constructed from 100 ng DNA and approximately 50-100K 300-base read pairs were generated on an Illumina MiSeq platform. GeneWiz Inc performed library preparation, sequencing and bioinformatics analysis.

A total of 66,473 reads were aligned to reference sequence. Sequences that occurred with the frequency of 5 or more were used for further analysis, and a total 63,526 sequences fit this criterion. Indels were detected in 62,864 sequence reads (~99%). There are 62,808 sequences with deletions. 42,334 reads (67%) harbor a 231 bp deletion between the two targets. 232 bp and 248 bp long deletions were also detected with lower frequencies (13.2%, and 1.2% respectively). There were 2399 sequences (~3.8%) with two deletions (12 bp and 19 bp) within the sgRNA1, and sgRNA2 respectively.

1   **Mice**

2   *Prox1*$^{+/Cre}$ (Srinivasan et al. 2010)*, Gata2*$^{f/f}$ (Charles et al. 2006)*, miR-126*$^{-/-}$ (Wang et al. 2008)*,*

3   *Prox1-CreERT2* (Srinivasan et al. 2007)*,* Tg(Prox1-tdTomato) (Gong et al. 2003)*, Lyve1-Cre*

4   (Pham et al. 2010)*,* Tie2-Cre (Kisanuki et al. 2001), *R26*$^{+/tdTomato}$ (Madisen et al. 2010) mice

5   were described previously. *Prox1*$^{+/Cre}$ mice were maintained in NMRI background. Other mice

6   were maintained in C57BL6 or C57BL6/NMRI mixed backgrounds. We used both male and

7   female mice for the experiments. All mice were housed and handled according to the

8   institutional IACUC protocols.

9

10   **miR-126 sponge and miR-126 over expression**

11   pSFFV plasmids to sequester miR-126 or over express miR-126 were reported previously

12   (Amendola et al. 2009; Gentner et al. 2009). Cyagen Bioscience (Santa Clara, CA, USA)

13   generated the lentiviral particles using LentiPAC 293 cells. HLECs were seeded at 50-60%

14   confluence on fribronectin-coated plates or glass slide. The following day cells are infected

15   with equal amounts of control, miR-126 sponge or miR-126 over expression virus for 4-6 hrs

16   according to manufacturer protocol using EBM2 medium and then changed to fresh medium.

17   After 2-3 days cells were harvested for appropriate study.

18

19   **miR-126 isolation and quantitative real-time PCR**

20   Micro RNA along with total RNA was isolated from HLECs using QIAzol lysis reagent (Qiagen,

21   Germantown, MD, USA) according to manufacturers instructions. The cDNA was synthesized

22   from total RNA (0.1 - 1.0 µg) with miScript II RT Kit (Qiagen, Germantown, MD, USA). qRT-

23   PCR was performed using miScript SYBR Green PCR Kit (Qiagen, Germantown, MD, USA) in

24   a CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). miR-126 Expression levels were

25

1 normalized to *U6.* Predesigned primers for miR-126 and U6 were purchased from Qiagen (cat

2 # 218300).

3

4 **RNA-seq analysis**

5 Total RNA was purified from HLECs infected with shGATA2 or control shRNA expressing

6 lentivirus particles. RNA was subjected to ribosomal RNA depletion followed by Truseq

7 stranded total RNA library preparation according to the manufacturer's instruction (Illumina).

8 RNA from miR-126-sponge treated HLEC-2 were processed using NEB Ultra II directional

9 RNA Library kit for Illumina. The resulting RNA-seq libraries were analyzed on the Illumina

10 HiSeq sequencing platform. The obtained sequencing reads were mapped with the bowtie2

11 algorithm using the RefSeq annotations (hg19 genome build) (Langmead and Salzberg 2012).

12 We utilized the RNA-seq analysis work flow within the Partek Genomics Suite (Partek

13 Incorporated) for quantitation and statistical analysis (ANOVA) of the transcriptome data. We

14 identified those transcripts that exhibited statistically significant differential expression in the

15 shGata2 samples compared to the shControl samples. We rank ordered the two lists based on

16 the expression level and magnitude of change. Using these rank-ordered list, we performed

17 gene ontology (GO) analysis for enriched biological terms (Eden et al. 2009). The genes

18 commonly regulated by GATA2 and miR-126 were analyzed using the functional annotation

19 platform of DAVID (Huang da et al. 2009a; Huang da et al. 2009b).

20

21 **Scanning Electron Microscopy**

22 SEM was performed according to our previous protocol (Geng et al. 2016; Geng and

23 Srinivasan 2018).

24

25

Statistical Analysis

For biochemical analysis n indicates the number of times the experiments were performed and for histological analysis n indicate the number of embryos analyzed per genotype. VE-Cadherin expression analysis in GATA2$^{\Delta HLEC}$ following miR-126 overexpression was performed twice. All other experiments were performed at least three times or more. Data were presented as mean ± SEM. GraphPad Prism 7 software was used to perform the statistical analysis. Data were analyzed by the unpaired, two-tailed, student's t test. P value < 0.05 was considered significant.

Western blot

Control HLEC or GATA2$^{\Delta HLEC}$ were grown in 12-well plates at ~ 100% confluency. Cells were harvested with lysis buffer and Western blots were performed using standard protocol.

1 **ACKNOWLEDGEMENT**

10

11 **DISCLOSURES**

12 None

13

14 **AUTHOR CONTRIBUTIONS**

15 MRM, XG, YCH, BC, LC, YK, GM, THK, K-CL and RSS performed experiments; DC, HC, Y-

16 KH, SC and JDE provided critical reagents; MRM, XG, and RSS designed the experiments

17 and wrote the manuscript; all authors provided input in designing the experiments and in

18 writing the manuscript.

19
20
21
22

REFERENCES

Amendola M, Passerini L, Pucci F, Gentner B, Bacchetta R, Naldini L. 2009. Regulated and multiple miRNA and siRNA delivery into primary cells by a lentiviral platform. *Molecular therapy : the journal of the American Society of Gene Therapy* **17**: 1039-1052.

Bertozzi CC, Schmaier AA, Mericko P, Hess PR, Zou Z, Chen M, Chen CY, Xu B, Lu MM, Zhou D et al. 2010. Platelets regulate lymphatic vascular development through CLEC-2-SLP-76 signaling. *Blood* **116**: 661-670.

Brouillard P, Boon L, Vikkula M. 2014. Genetics of lymphatic anomalies. *The Journal of clinical investigation* **124**: 898-904.

Castorena-Gonzalez JA, Zawieja SD, Li M, Srinivasan RS, Simon AM, de Wit C, de la Torre R, Martinez-Lemus LA, Hennig GW, Davis MJ. 2018. Mechanisms of Connexin-Related Lymphedema. *Circulation research* **123**: 964-985.

Cha B, Geng X, Mahamud MR, Fu J, Mukherjee A, Kim Y, Jho EH, Kim TH, Kahn ML, Xia L et al. 2016. Mechanotransduction activates canonical Wnt/beta-catenin signaling to promote lymphatic vascular patterning and the development of lymphatic and lymphovenous valves. *Genes Dev* **30**: 1454-1469.

Cha B, Geng X, Mahamud MR, Zhang JY, Chen L, Kim W, Jho EH, Kim Y, Choi D, Dixon JB et al. 2018. Complementary Wnt Sources Regulate Lymphatic Vascular Development via PROX1-Dependent Wnt/beta-Catenin Signaling. *Cell reports* **25**: 571-584 e575.

Charles MA, Saunders TL, Wood WM, Owens K, Parlow AF, Camper SA, Ridgway EC, Gordon DF. 2006. Pituitary-specific Gata2 knockout: effects on gonadotrope and thyrotrope function. *Molecular endocrinology (Baltimore, Md)* **20**: 1366-1377.

Charpentier MS, Christine KS, Amin NM, Dorr KM, Kushner EJ, Bautch VL, Taylor JM, Conlon FL. 2013. CASZ1 promotes vascular assembly and morphogenesis through the direct regulation of an EGFL7/RhoA-mediated pathway. *Developmental cell* **25**: 132-143.

Chen H, Griffin C, Xia L, Srinivasan RS. 2014. Molecular and cellular mechanisms of lymphatic vascular maturation. *Microvasc Res* **96**: 16-22.

Choi D, Park E, Jung E, Cha B, Lee S, Yu J, Kim PM, Lee S, Hong YJ, Koh CJ et al. 2019. Piezo1 incorporates mechanical force signals to genetic program that governs lymphatic valve development and maintenance. *JCI Insight*.

Chrzanowska-Wodnicka M. 2017. Rap1 in endothelial biology. *Curr Opin Hematol* **24**: 248-255.

Craven SE, Lim KC, Ye W, Engel JD, de Sauvage F, Rosenthal A. 2004. Gata2 specifies serotonergic neurons downstream of sonic hedgehog. *Development* **131**: 1165-1173.

Crispino JD, Horwitz MS. 2017. GATA factor mutations in hematologic disease. *Blood* **129**: 2103-2110.

Dellinger M, Hunter R, Bernas M, Gale N, Yancopoulos G, Erickson R, Witte M. 2008. Defective remodeling and maturation of the lymphatic vasculature in Angiopoietin-2 deficient mice. *Developmental biology* **319**: 309-320.

Donadieu J, Lamant M, Fieschi C, de Fontbrune FS, Caye A, Ouachee M, Beaupain B, Bustamante J, Poirel HA, Isidor B et al. 2018. Natural history of GATA2 deficiency in a survey of 79 French and Belgian patients. *Haematologica* **103**: 1278-1287.

Dudek SM, Garcia JG. 2001. Cytoskeletal regulation of pulmonary vascular permeability. *J Appl Physiol (1985)* **91**: 1487-1500.

Eden E, Navon R, Steinfeld I, Lipson D, Yakhini Z. 2009. GOrilla: a tool for discovery and visualization of enriched GO terms in ranked gene lists. *BMC Bioinformatics* **10**: 48.

Emberger JM, Navarro M, Dejean M, Izarn P. 1979. [Deaf-mutism, lymphedema of the lower limbs and hematological abnormalities (acute leukemia, cytopenia) with autosomal dominant transmission]. *J Genet Hum* **27**: 237-245.

Fish JE, Santoro MM, Morton SU, Yu S, Yeh RF, Wythe JD, Ivey KN, Bruneau BG, Stainier DY, Srivastava D. 2008. miR-126 regulates angiogenic signaling and vascular integrity. *Developmental cell* **15**: 272-284.

Frye M, Taddei A, Dierkes C, Martinez-Corral I, Fielden M, Ortsater H, Kazenwadel J, Calado DP, Ostergaard P, Salminen M et al. 2018. Matrix stiffness controls lymphatic vessel formation through regulation of a GATA2-dependent transcriptional program. *Nature communications* **9**: 1511.

Fu J, Gerhardt H, McDaniel JM, Xia B, Liu X, Ivanciu L, Ny A, Hermans K, Silasi-Mansat R, McGee S et al. 2008. Endothelial cell O-glycan deficiency causes blood/lymphatic misconnections and consequent fatty liver disease in mice. *The Journal of clinical investigation* **118**: 3725-3737.

Gale NW, Thurston G, Hackett SF, Renard R, Wang Q, McClain J, Martin C, Witte C, Witte MH, Jackson D et al. 2002. Angiopoietin-2 is required for postnatal angiogenesis and lymphatic patterning, and only the latter role is rescued by Angiopoietin-1. *Developmental cell* **3**: 411-423.

Geng X, Cha B, Mahamud MR, Lim KC, Silasi-Mansat R, Uddin MK, Miura N, Xia L, Simon AM, Engel JD et al. 2016. Multiple mouse models of primary lymphedema exhibit distinct defects in lymphovenous valve development. *Developmental biology* **409**: 218-233.

Geng X, Srinivasan RS. 2018. Correlative Fluorescence and Scanning Electron Microscopy to Study Lymphovenous Valve Development. *Methods in molecular biology* **1846**: 85-96.

Gentner B, Schira G, Giustacchini A, Amendola M, Brown BD, Ponzoni M, Naldini L. 2009. Stable knockdown of microRNA in vivo by lentiviral vectors. *Nat Methods* **6**: 63-66.

Gong S, Zheng C, Doughty ML, Losos K, Didkovsky N, Schambra UB, Nowak NJ, Joyner A, Leblanc G, Hatten ME et al. 2003. A gene expression atlas of the central nervous system based on bacterial artificial chromosomes. *Nature* **425**: 917-925.

Hagerling R, Hoppe E, Dierkes C, Stehling M, Makinen T, Butz S, Vestweber D, Kiefer F. 2018. Distinct roles of VE-cadherin for development and maintenance of specific lymph vessel beds. *The EMBO journal* **37**.

Hahn C, Schwartz MA. 2009. Mechanotransduction in vascular physiology and atherogenesis. *Nature reviews Molecular cell biology* **10**: 53-62.

Hartmann D, Fiedler J, Sonnenschein K, Just A, Pfanne A, Zimmer K, Remke J, Foinquinos A, Butzlaff M, Schimmel K et al. 2016. MicroRNA-Based Therapy of GATA2-Deficient Vascular Disease. *Circulation* **134**: 1973-1990.

Huang da W, Sherman BT, Lempicki RA. 2009a. Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic acids research* **37**: 1-13.

-. 2009b. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature protocols* **4**: 44-57.

Ingber DE. 2006. Cellular mechanotransduction: putting all the pieces together again. *FASEB J* **20**: 811-827.

Janardhan HP, Milstone ZJ, Shin M, Lawson ND, Keaney JF, Jr., Trivedi CM. 2017. Hdac3 regulates lymphovenous and lymphatic valve formation. *The Journal of clinical investigation* **127**: 4193-4206.

Kazenwadel J, Betterman KL, Chong CE, Stokes PH, Lee YK, Secker GA, Agalarov Y, Demir CS, Lawrence DM, Sutton DL et al. 2015. GATA2 is required for lymphatic vessel valve development and maintenance. *The Journal of clinical investigation* **125**: 2979-2994.

Kazenwadel J, Secker GA, Liu YJ, Rosenfeld JA, Wildin RS, Cuellar-Rodriguez J, Hsu AP, Dyack S, Fernandez CV, Chong CE et al. 2012. Loss-of-function germline GATA2

mutations in patients with MDS/AML or MonoMAC syndrome and primary lymphedema reveal a key role for GATA2 in the lymphatic vasculature. *Blood* **119**: 1283-1291.

Khandekar M, Brandt W, Zhou Y, Dagenais S, Glover TW, Suzuki N, Shimizu R, Yamamoto M, Lim KC, Engel JD. 2007. A Gata2 intronic enhancer confers its pan-endothelia-specific regulation. *Development* **134**: 1703-1712.

Kisanuki YY, Hammer RE, Miyazaki J, Williams SC, Richardson JA, Yanagisawa M. 2001. Tie2-Cre transgenic mice: a new model for endothelial cell-lineage analysis in vivo. *Developmental biology* **230**: 230-242.

Kontarakis Z, Rossi A, Ramas S, Dellinger MT, Stainier DYR. 2018. Mir-126 is a conserved modulator of lymphatic development. *Developmental biology*.

Kuhnert F, Mancuso MR, Hampton J, Stankunas K, Asano T, Chen CZ, Kuo CJ. 2008. Attribution of vascular phenotypes of the murine Egfl7 locus to the microRNA miR-126. *Development* **135**: 3989-3993.

Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. 2002. Identification of tissue-specific microRNAs from mouse. *Current biology : CB* **12**: 735-739.

Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9**: 357-359.

Lechman ER, Gentner B, van Galen P, Giustacchini A, Saini M, Boccalatte FE, Hiramatsu H, Restuccia U, Bachi A, Voisin V et al. 2012. Attenuation of miR-126 activity expands HSC in vivo without exhaustion. *Cell stem cell* **11**: 799-811.

Lim KC, Hosoya T, Brandt W, Ku CJ, Hosoya-Ohmura S, Camper SA, Yamamoto M, Engel JD. 2012. Conditional Gata2 inactivation results in HSC loss and lymphatic mispatterning. *The Journal of clinical investigation* **122**: 3705-3717.

Madisen L, Zwingman TA, Sunkin SM, Oh SW, Zariwala HA, Gu H, Ng LL, Palmiter RD, Hawrylycz MJ, Jones AR et al. 2010. A robust and high-throughput Cre reporting and characterization system for the whole mouse brain. *Nat Neurosci* **13**: 133-140.

Mammoto A, Connor KM, Mammoto T, Yung CW, Huh D, Aderman CM, Mostoslavsky G, Smith LE, Ingber DE. 2009. A mechanosensitive transcriptional mechanism that controls angiogenesis. *Nature* **457**: 1103-1108.

Muzumdar MD, Tasic B, Miyamichi K, Li L, Luo L. 2007. A global double-fluorescent Cre reporter mouse. *Genesis* **45**: 593-605.

Nonomura K, Lukacs V, Sweet DT, Goddard LM, Kanie A, Whitwam T, Ranade SS, Fujimori T, Kahn ML, Patapoutian A. 2018. Mechanically activated ion channel PIEZO1 is required for lymphatic valve formation. *Proceedings of the National Academy of Sciences of the United States of America*.

Ostergaard P, Simpson MA, Connell FC, Steward CG, Brice G, Woollard WJ, Dafou D, Kilo T, Smithson S, Lunt P et al. 2011. Mutations in GATA2 cause primary lymphedema associated with a predisposition to acute myeloid leukemia (Emberger syndrome). *Nature genetics* **43**: 929-931.

Parker LH, Schmidt M, Jin SW, Gray AM, Beis D, Pham T, Frantz G, Palmieri S, Hillan K, Stainier DY et al. 2004. The endothelial-cell-derived secreted factor Egfl7 regulates vascular tube formation. *Nature* **428**: 754-758.

Pham TH, Baluk P, Xu Y, Grigorova I, Bankovich AJ, Pappu R, Coughlin SR, McDonald DM, Schwab SR, Cyster JG. 2010. Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning. *The Journal of experimental medicine* **207**: 17-27.

Renier N, Wu Z, Simon DJ, Yang J, Ariel P, Tessier-Lavigne M. 2014. iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. *Cell* **159**: 896-910.

31

1   Rupaimoole R, Slack FJ. 2017. MicroRNA therapeutics: towards a new era for the
2   management of cancer and other diseases. *Nature reviews Drug discovery* **16**: 203-
3   222.
4   Sabine A, Agalarov Y, Maby-El Hajjami H, Jaquet M, Hagerling R, Pollmann C, Bebber D,
5   Pfenniger A, Miura N, Dormond O et al. 2012. Mechanotransduction, PROX1, and
6   FOXC2 cooperate to control connexin37 and calcineurin during lymphatic-valve
7   formation. *Developmental cell* **22**: 430-445.
8   Souma T, Thomson BR, Heinen S, Carota IA, Yamaguchi S, Onay T, Liu P, Ghosh AK, Li C,
9   Eremina V et al. 2018. Context-dependent functions of angiopoietin 2 are determined by
10   the endothelial phosphatase VEPTP. *Proceedings of the National Academy of Sciences*
11   *of the United States of America* **115**: 1298-1303.
12 Spinner MA, Sanchez LA, Hsu AP, Shaw PA, Zerbe CS, Calvo KR, Arthur DC, Gu W, Gould
13   CM, Brewer CC et al. 2014. GATA2 deficiency: a protean disorder of hematopoiesis,
14   lymphatics, and immunity. *Blood* **123**: 809-821.
15 Srinivasan RS, Dillard ME, Lagutin OV, Lin FJ, Tsai S, Tsai MJ, Samokhvalov IM, Oliver G.
16   2007. Lineage tracing demonstrates the venous origin of the mammalian lymphatic
17   vasculature. *Genes Dev* **21**: 2422-2432.
18 Srinivasan RS, Geng X, Yang Y, Wang Y, Mukatira S, Studer M, Porto MP, Lagutin O, Oliver
19   G. 2010. The nuclear hormone receptor Coup-TFII is required for the initiation and early
20   maintenance of Prox1 expression in lymphatic endothelial cells. *Genes Dev* **24**: 696-
21   707.
22 Srinivasan RS, Oliver G. 2011. Prox1 dosage controls the number of lymphatic endothelial cell
23   progenitors and the formation of the lymphovenous valves. *Genes Dev* **25**: 2187-2197.
24 Sweet DT, Jimenez JM, Chang J, Hess PR, Mericko-Ishizuka P, Fu J, Xia L, Davies PF, Kahn
25   ML. 2015. Lymph flow regulates collecting lymphatic vessel maturation in vivo. *The*
26   *Journal of clinical investigation* **125**: 2995-3007.
27 Tammela T, Alitalo K. 2010. Lymphangiogenesis: Molecular mechanisms and future promise.
28   *Cell* **140**: 460-476.
29 Van Roosbroeck K, Pollet J, Calin GA. 2013. miRNAs and long noncoding RNAs as
30   biomarkers in human diseases. *Expert review of molecular diagnostics* **13**: 183-204.
31 Wang S, Aurora AB, Johnson BA, Qi X, McAnally J, Hill JA, Richardson JA, Bassel-Duby R,
32   Olson EN. 2008. The endothelial-specific microRNA miR-126 governs vascular integrity
33   and angiogenesis. *Developmental cell* **15**: 261-271.
34 Zhou Q, Anderson C, Hanus J, Zhao F, Ma J, Yoshimura A, Wang S. 2016. Strand and Cell
35   Type-specific Function of microRNA-126 in Angiogenesis. *Molecular therapy : the*
36   *journal of the American Society of Gene Therapy* **24**: 1823-1835.
37 Zhou Y, Lim KC, Onodera K, Takahashi S, Ohta J, Minegishi N, Tsai FY, Orkin SH, Yamamoto
38   M, Engel JD. 1998. Rescue of the embryonic lethal hematopoietic defect reveals a
39   critical role for GATA-2 in urogenital development. *The EMBO journal* **17**: 6689-6700.
40 Zhou Y, Yamamoto M, Engel JD. 2000. GATA2 is required for the generation of V2
41   interneurons. *Development* **127**: 3829-3838.
42
43

1 **FIGURES AND FIGURE LEGENDS**

2   **Figure 1**
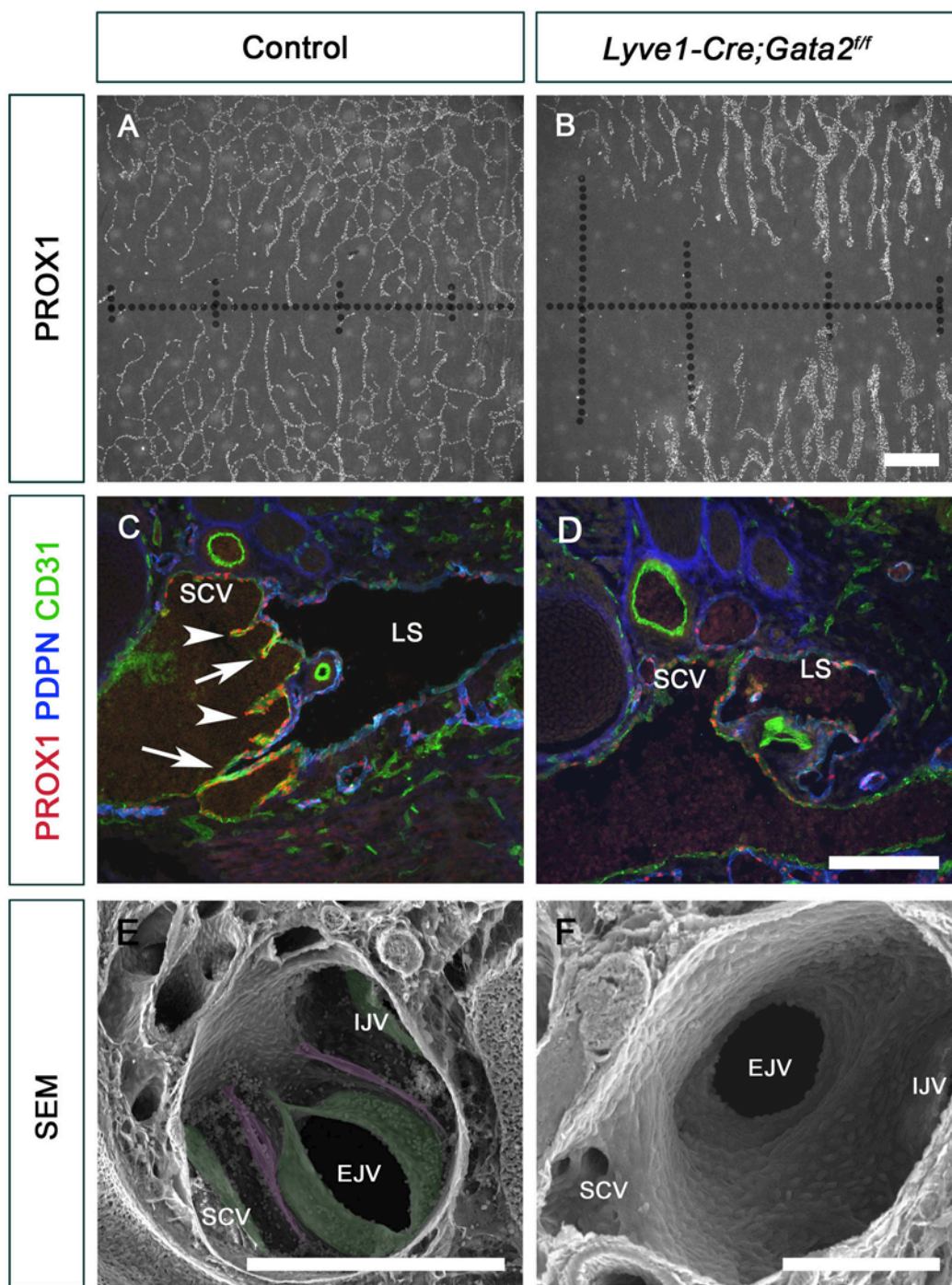
3
4

1 **Figure 1: Lymphatic vessels are defective and LVVs are absent in *Lyve-Cre;Gata2^{f/f}***
2 **embryos.**
3
4 E16.5 control and *Lyve-Cre;Gata2^{f/f}* littermates were analyzed. (A, B) The lymphatic vessels in
5 the dorsal skin of mutants were hypoplastic, dilated and had fewer branch points. (C, D) LVVs
6 (arrows) and VVs (arrowheads) were seen in control, but not in mutants (D). (E, F) SEM
7 confirmed that LVVs (magenta) and VVs (green) were present in control but not in mutant
8 embryos.
9
10 Abbreviations: IJV, internal jugular vein; EJV, external jugular vein; SCV, subclavian vein; LS,
11 lymph sac.
12
13 Measuring bar: (A-B) 500 μm; (C-D) 200 μm; (E) 300 μm; (F) 100 μm.
14
15 Statistics: (A-B) n= 3 embryos; (C-D) n= 3 embryos and 6 LVVs per genotype; (E-F) n= 3
16 embryos and 5 LVV complexes per genotype.
17

34

1
2
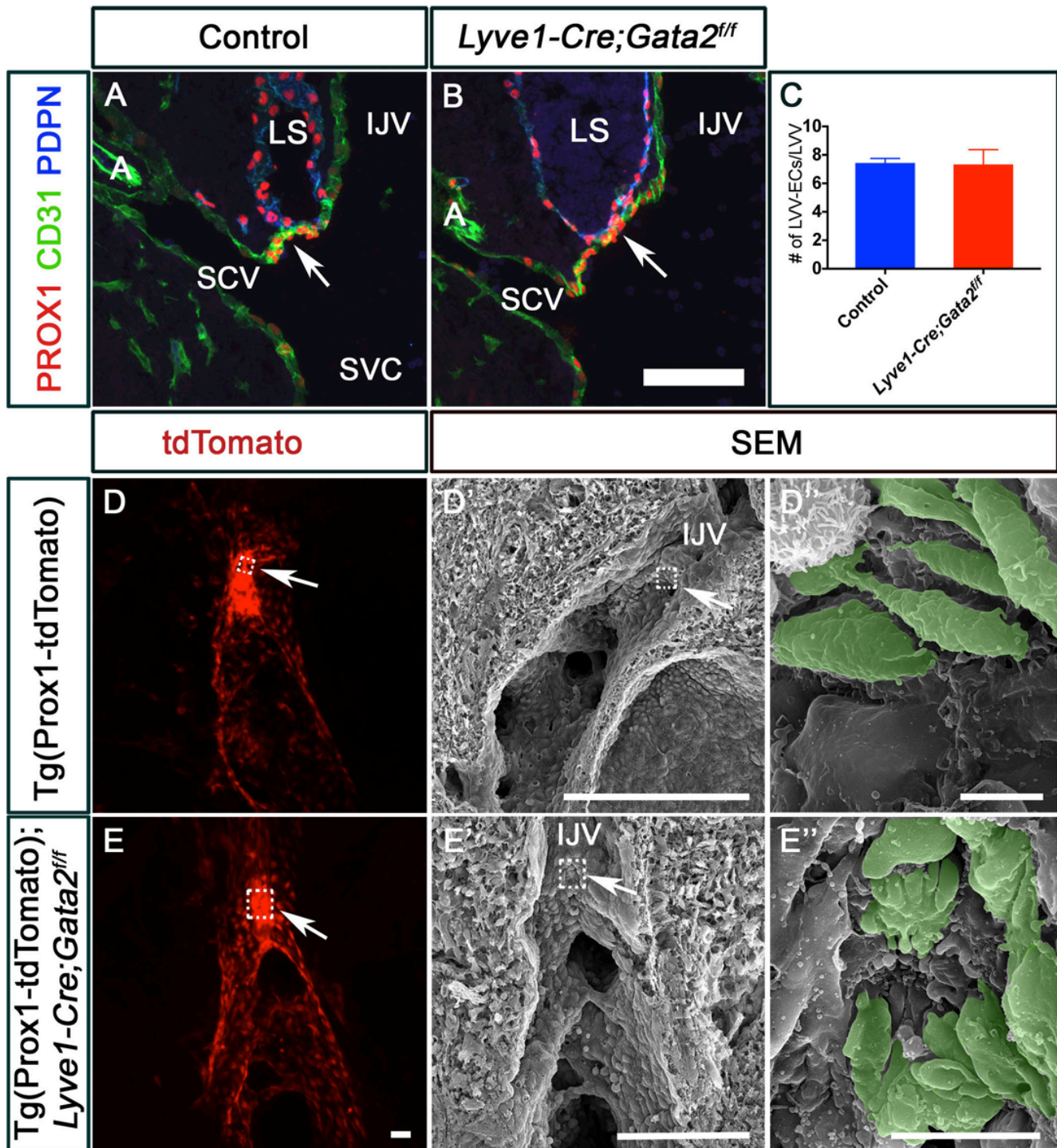
Figure 2

3
4

1 **Figure 2: GATA2 is required for the proper architecture of lymphovenous valve-forming**
2 **endothelial cells (LVV-ECs).**
3
4 (A-C) PROX1^{high} LVV-ECs were observed in both E12.0 control (A) and *Lyve1-Cre;Gata2$^{f/f}$* (B)
5 embryos. Blood cells were seen within the lymph sacs (LS) of mutant embryos. (C) No obvious
6 difference in LVV-EC numbers was observed between the two genotypes.
7
8 (D, E) E12.0 Tg(Prox1-tdTomato) (D) and Tg(Prox1-tdTomato); *Lyve1-Cre;Gata2$^{f/f}$* (E)
9 embryos were sagitally sectioned along the internal jugular vein (IJV). The fluorescent signal
10 from the reporter revealed LVV-ECs in both control and mutant embryos. (D', E') The samples
11 from D and E were analyzed using SEM, which revealed the LVV-ECs (pseudo-colored in
12 green) with elongated morphology in control embryos (D' and magnified figure of the boxed
13 region in D''). In contrast, the LVV-ECs of mutant embryos were dysplastic (E' and E'').
14
15 Abbreviations: A, artery; IJV, internal jugular vein; SCV, subclavian vein; SVC, superior vena
16 cava; LS, lymph sac.
17
18 Measuring bar: (A, B) 100 µm; (D, E) 50 µm; (D') 200 µm; (D'') 5 µm; (E') 100 µm; (E'') 10 µm.
19
20 Statistics: (A-E) n= 3 embryos and 6 LVV complexes per genotype per stage.
21

1
2

Figure 2- Figure Supplement 1
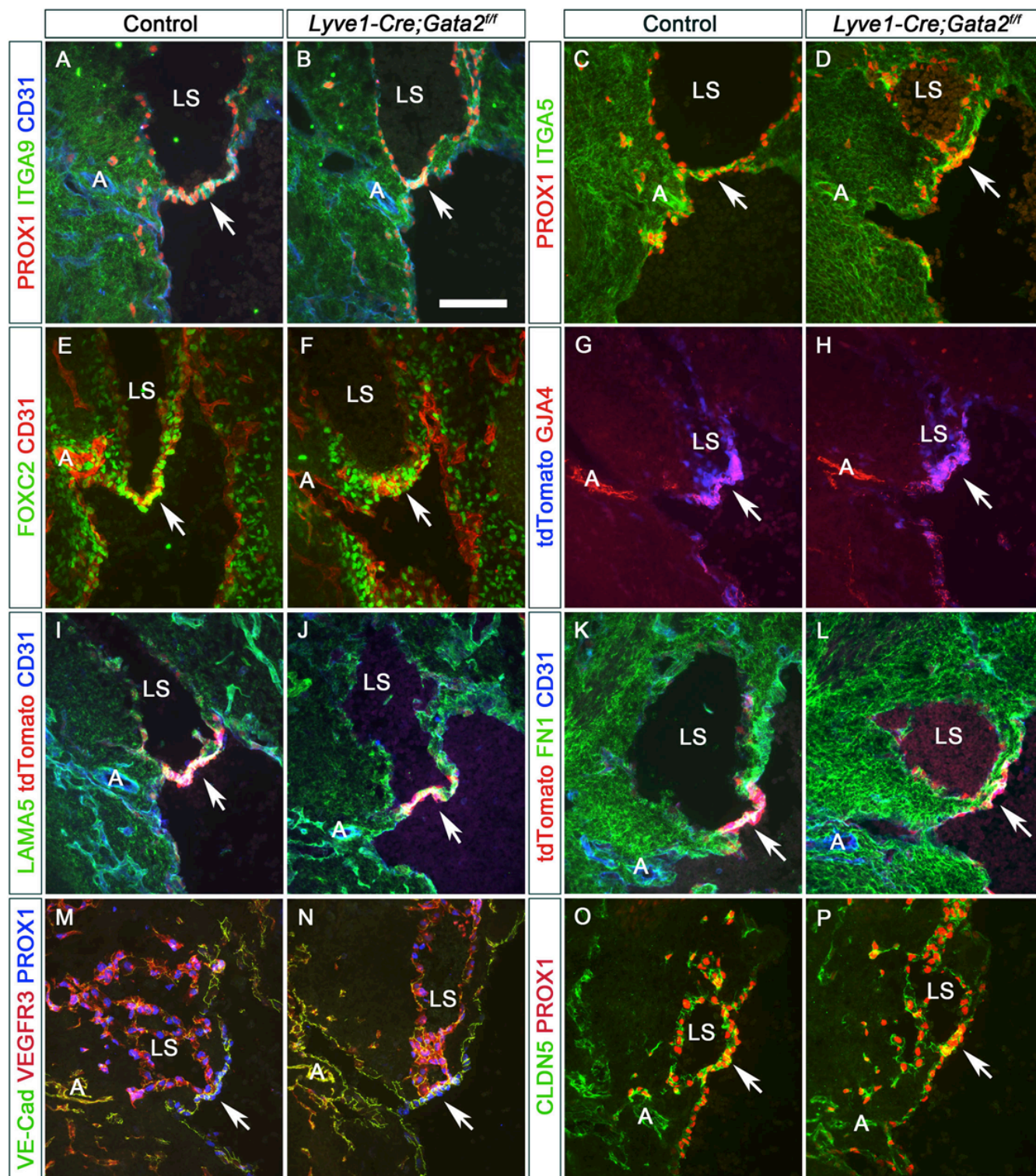
3
4

1 **Figure 2- Figure Supplement 1: LVV-ECs, ECM and cell junction molecules are normally**
2 **expressed in E12.0 embryos lacking GATA2.**
3
4 E12.0 Wild type and *Lyve1-Cre;Gata2^{f/f}* littermates or Tg(Prox1-tdTomato) and Tg(Prox1-
5 tdTomato); *Lyve1-Cre;Gata2^{f/f}* littermates were frontally sectioned and IHC was performed for
6 LVV-EC markers (PROX1, tdTomato, FOXC2, ITGA5, ITGA9), ECM components (FN1,
7 LAMA5), and cell junction proteins (CD31, VE-Cadherin, GJA4, CLDN5). No obvious
8 differences were observed between control and mutant samples.
9
10 Abbreviations: A, artery; LS, lymph sac.
11
12 Measuring bar: (A-P) 100 μm.
13
14 Statistics: n=3 embryos and 6 LVV complexes per genotype per antibody.
15
16

1 **Figure 2- Figure Supplement 2**
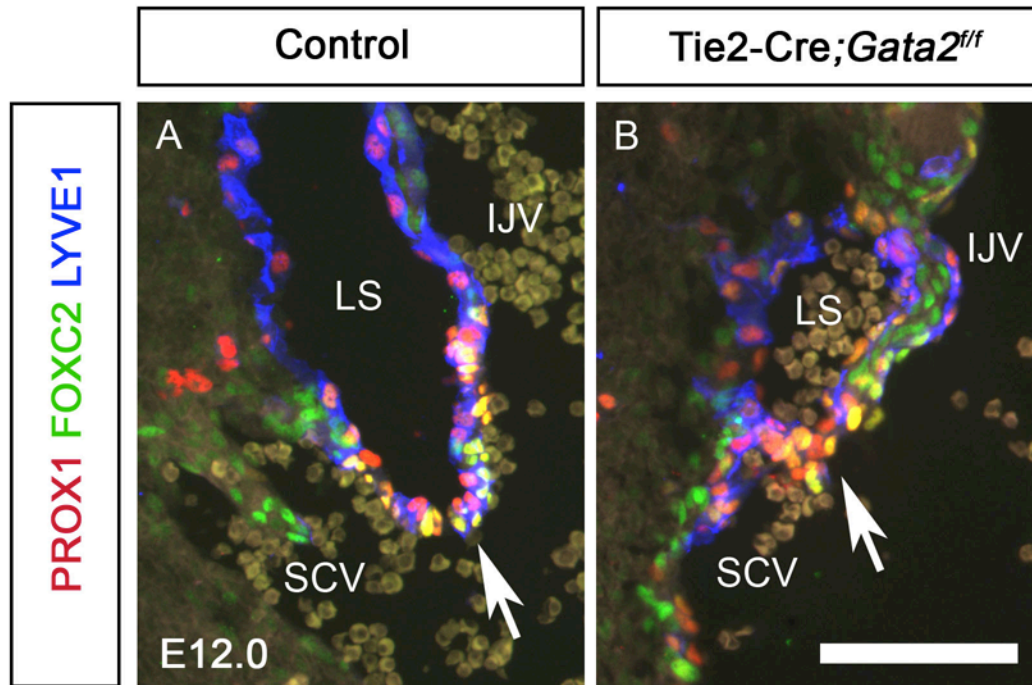
2
3
4 **Figure 2- Figure Supplement 2: LVV-ECs are present in E12.0 Tie2-Cre;*Gata2^{f/f}* embryos**
5
6 PROX1$^+$FOXC2$^+$ LVV-ECs are present in both E12.0 control (A, arrow) and mutant embryos
7 lacking GATA2 in all endothelial cells (B, arrow).
8
9 Abbreviations: IJV, internal jugular vein; SCV, subclavian vein; LS, lymph sac.
10
11 Measuring bar: (A, B) 100 µm.
12
13 Statistics: n=3 embryos and 6 LVV complexes per genotype.
14
15

1
2

Figure 2- Figure Supplement 3
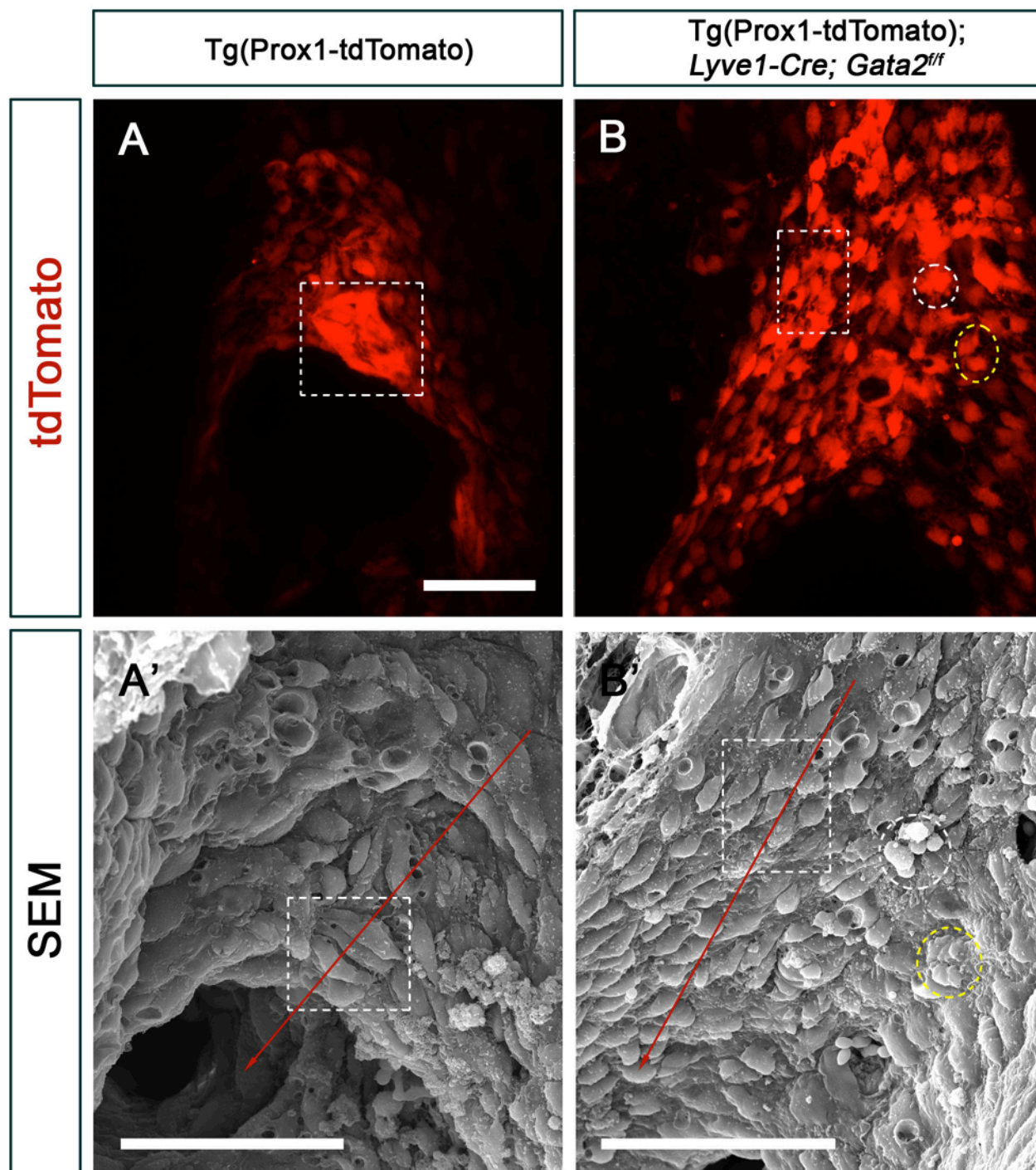
3

1 **Figure 2- Figure Supplement 3: LVV-ECs of E12.0 *Gata2^{LECKO}* embryos are dysplastic**
2 **and do not align properly with respect to blood flow**
3
4 (A, B) E12.0 Tg(Prox1-tdTomato) or Tg(Prox1-tdTomato); *Lyve1-Cre; Gata2$^{f/f}$* embryos were
5 sagittally sectioned along the internal jugular vein and the tdTomato$^+$ LVV-ECs were imaged
6 by confocal microscopy. The same samples were then reprocessed and analyzed by SEM (A',
7 B'). The cells in fluorescent microscopy images that correlate with cells in the SEM images are
8 outlined.
9
10 (A') LVV-ECs of control embryos were elongated and compactly aggregated with each other.
11 They were also aligned perpendicular to the direction of blood flow (red arrow). (B') In contrast,
12 LVV-ECs of mutant embryos appear round and dispersed (see cells within the rectangular box)
13 and appeared to be aligned parallel to the flow. In addition, several dysplastic cells were also
14 seen (within white and yellow circles).
15
16 Measuring bar: (A-B) 50 µm; (A') 40 µm; (B') 50 µm.
17
18 Statistics: n=3 embryos, 6 LVV complexes per genotype.
19
20

1 **Figure 3**

2
3

42
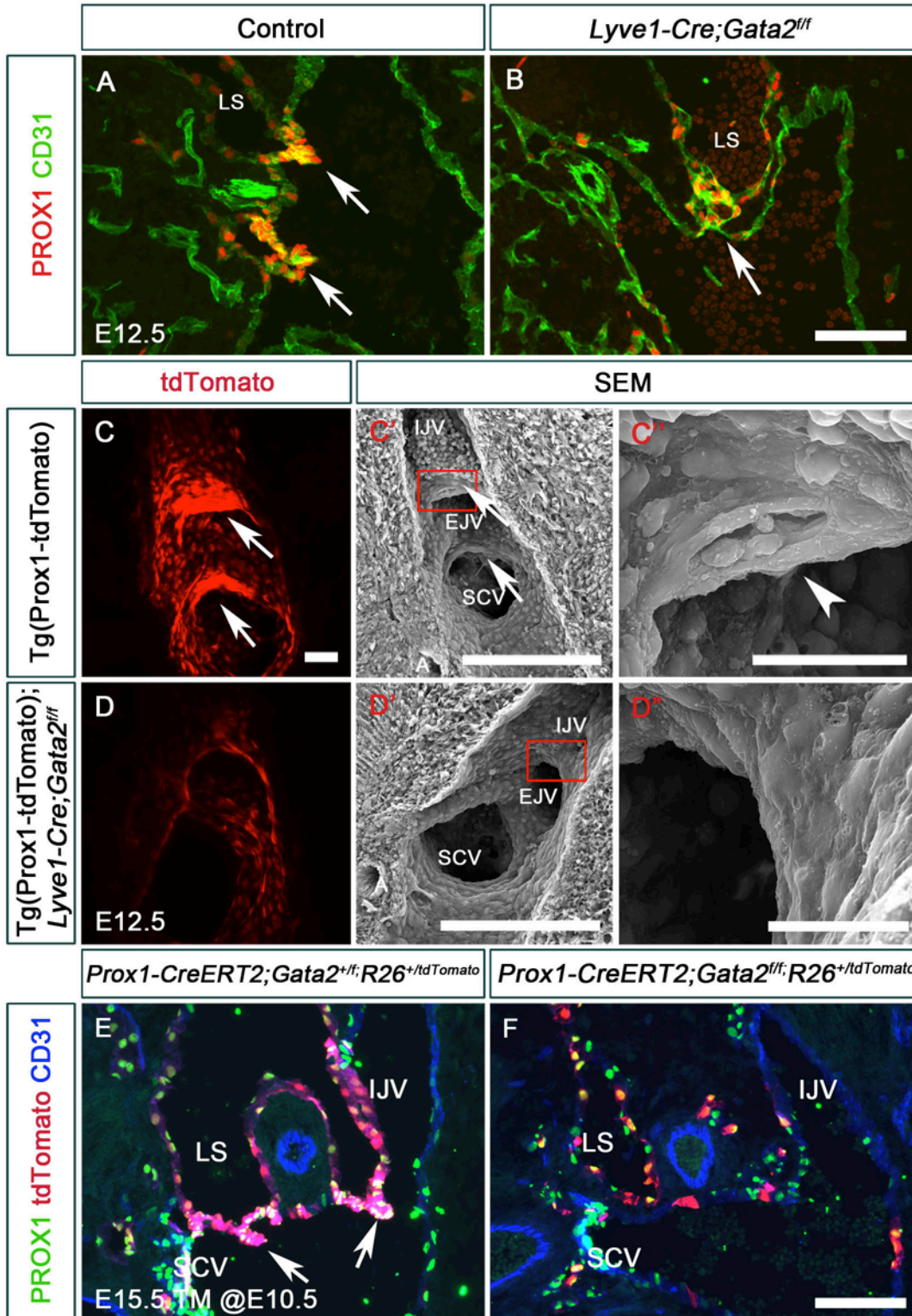
1 **Figure 3: LVV-ECs are lost from E12.5 embryos lacking GATA2.**
2
3 (A, B) E12.5 wild type and *Lyve1-Cre;Gata2$^{f/f}$* embryos were analyzed by
4 immunohistochemistry on sections. LVV-ECs had invaginated into the vein in control embryos
5 (A, arrows). In contrast, very few PROX1$^+$ cells were observed in mutant embryos (B, arrow).
6
7 (C, D) E12.5 Tg(Prox1-tdTomato) and Tg(Prox1-tdTomato); *Lyve1-Cre;Gata2$^{f/f}$* embryos were
8 sagitally sectioned along the internal jugular vein (IJV) and the fluorescent signal was analyzed
9 using confocal microscopy. LVVs with strong tdTomato signal were seen in control (C, arrows),
10 but not in mutant (D) embryos. (C', C'', D', D'') The samples from C and D were re-processed
11 and analyzed by SEM. In controls, LVV-ECs (arrows) with elongated morphology were
12 observed at the junction of IJV and external jugular vein (EJV) and at the junction of IJV and
13 subclavian vein (SCV). An opening connecting the lymph and blood circulations was also seen
14 (arrowhead in C''). In contrast, endothelial cells at the junction of veins were indistinguishable
15 from the rest of the venous endothelial cells in mutant embryos (D', D'').
16
17 (E, F) Lineage tracing was performed using *Prox1-CreERT2;R26$^{+/tdTomato}$* in *Gata2$^{+/f}$* (E) or
18 *Gata2$^{f/f}$* (F) backgrounds. Tamoxifen was injected at E10.5 to label the PROX1$^+$ LVV-ECs and
19 LECs with tdTomato. Subsequently, the embryos were analyzed at E15.5. While LVV-ECs
20 were well labeled in control embryos (E, arrows), LVVs were absent and very few labeled cells
21 were observed in the veins of mutant embryos (F).
22
23 Abbreviations: IJV, internal jugular vein; SCV, subclavian vein; SVC, superior vena cava; LS,
24 lymph sac.
25
26 Measuring bar: (A,B, E, F) 100 µm; (C, D) 50 µm; (C') 200 µm; (D') 500 µm; (C'', D'') 30 µm.
27
28 Statistics: (A-B) n=6 embryos per genotype; (C-D) n= 3 embryos and 6 LVV complexes per
29 genotype; (E, F) n=3 embryos per genotype.
30
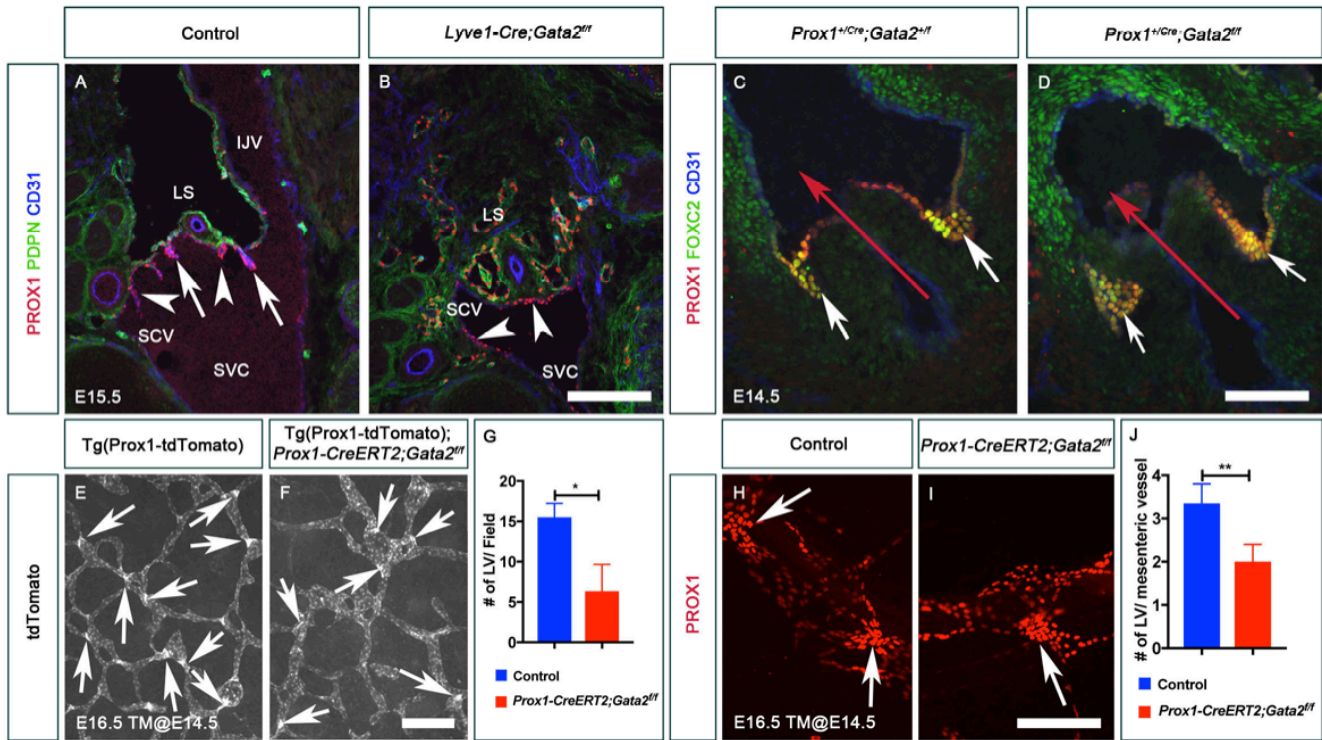31
32

43

1   **Figure 4**

Figure 4: GATA2 is not required for the differentiation of venous valve-, cardiac valve-, and lymphatic valve-forming endothelial cells.

(A) LVVs (arrows) and venous valves (VVs, arrowheads) were seen at the junction of internal jugular vein (IJV), subclavian vein (SCV) and superior vena cava (SVC) of E15.5 control embryos. (B) LVVs were absent in E15.5 *Lyve1-Cre;Gata2^{f/f}* embryos. While VV-ECs were present in the mutants (arrowheads), they did not invaginate into the veins and did not have the proper morphology of VVs.

(C, D) PROX1+ FOXC2+ endothelial cells were observed on the downstream side of cardiac valves in both control (C) and *Prox1^{+/Cre};Gata2^{f/f}* (D) embryos, which lack GATA2 in all PROX1-expressing cells. The red arrow indicates the direction of blood flow.

(E-J) Lymphatic vessels in the dorsal skin (E-G) and mesentery (H-J) of E16.5 control and *Prox1-CreERT2;Gata2^{f/f}* embryos, which were exposed to tamoxifen at E14.5, were analyzed. Lymphatic valve-forming endothelial cells (LV-ECs) were seen in both control and mutant embryos (arrows). However, LV clusters are reduced in mutants (G, J).

Abbreviations: IJV, internal jugular vein; SCV, subclavian vein; SVC, superior vena cava; LS, lymph sac.

Measuring bar: (A, B, H, I) 200 μm; (D) 100 μm; (E, F) 250 μm.

Statistics: (A-B) n= 3 embryos and 6 LVV complexes per genotype; (C-D) n= 3 embryos per genotype; (E-F, H-I) n= 3 embryos per genotype. (**) p<0.01; (*) p<0.05.

44

Figure 5

1
2

3
4
5
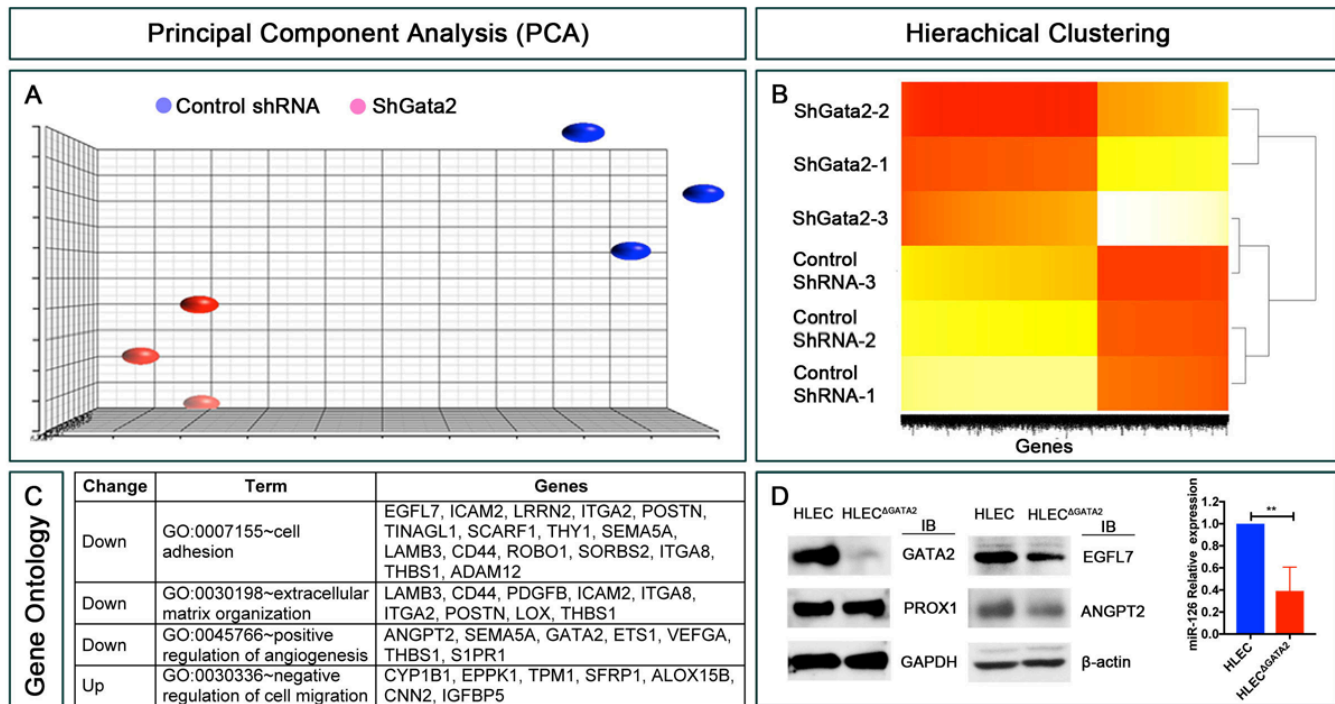
Figure 5: RNA-seq identifies the targets of GATA2 in primary human LECs.

(A) Principal component analysis (PCA) was performed on RNA-seq data from control shRNA and shGATA2 infected primary human lymphatic endothelial cells (HLECs). A high level of similarity was observed within the groups as indicated by their proximity to each other. (B) Hierarchical clustering shows that approximately 1000 genes were consistently downregulated and 600 genes were upregulated in shGATA2 treated HLECs. (C) GO revealed a list of genes that are likely relevant to the phenotypes observed mice lacking GATA2. (D) GATA2 was knocked out from a second HLEC line using CRISPR/Cas9. Western blot revealed the lack of GATA2 and the downregulation of EGFL7 and ANGPT2 in the knock out cells (HLEC$^{\Delta GATA2}$). In contrast, no obvious differences were observed in the expression of PROX1. Additionally, qRT-PCR revealed the downregulation of miR-126.

Statistics: (A) n=3 independent experiments per shRNA; (D) n= 3 independent experiments (antibiotic selection, Western blot and qRT-PCR). (**) p<0.01

1 **Figure 5- Figure Supplement 1**

2

GATA2 (chr3:128,479,427-128,493,185)
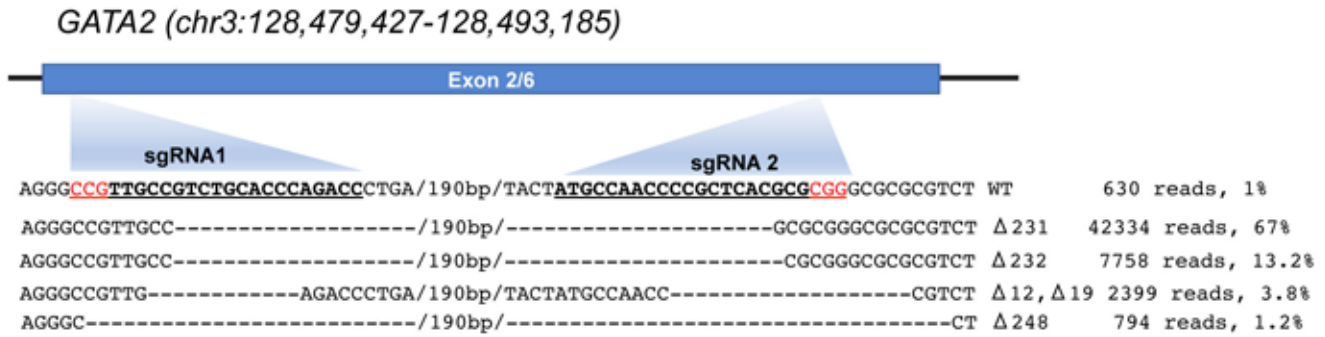
3
4
5 **Figure 5- Figure Supplement 1: Efficient deletion of GATA2 from HLECs by**
6 **CRISPR/Cas9.**
7
8 The second exon (out of six total exons) of *GATA2* is schematically shown. Sequences
9 corresponding to the sgRNAs are in bold and are underlined. The PAM sequences are in red.
10 The ATG of GATA2 is located in between sgRNA1 and sgRNA2. HLEC-2 were infected with
11 lentiviruses expressing Cas9 and the two sgRNAs and selected using puromycin. The region
12 between sgRNA1 and sgRNA2 was PCR amplified and was sequenced using NGS approach.
13 The sequence and contribution of major indels is depicted. These results demonstrate that
14 ~99% of DNA fragments have large deletions in exon 2 of *GATA2*.
15
16 Statistics: Sequencing was performed using one batch of selected cells (out of 3 independently
17 selected batches).
18

1
2
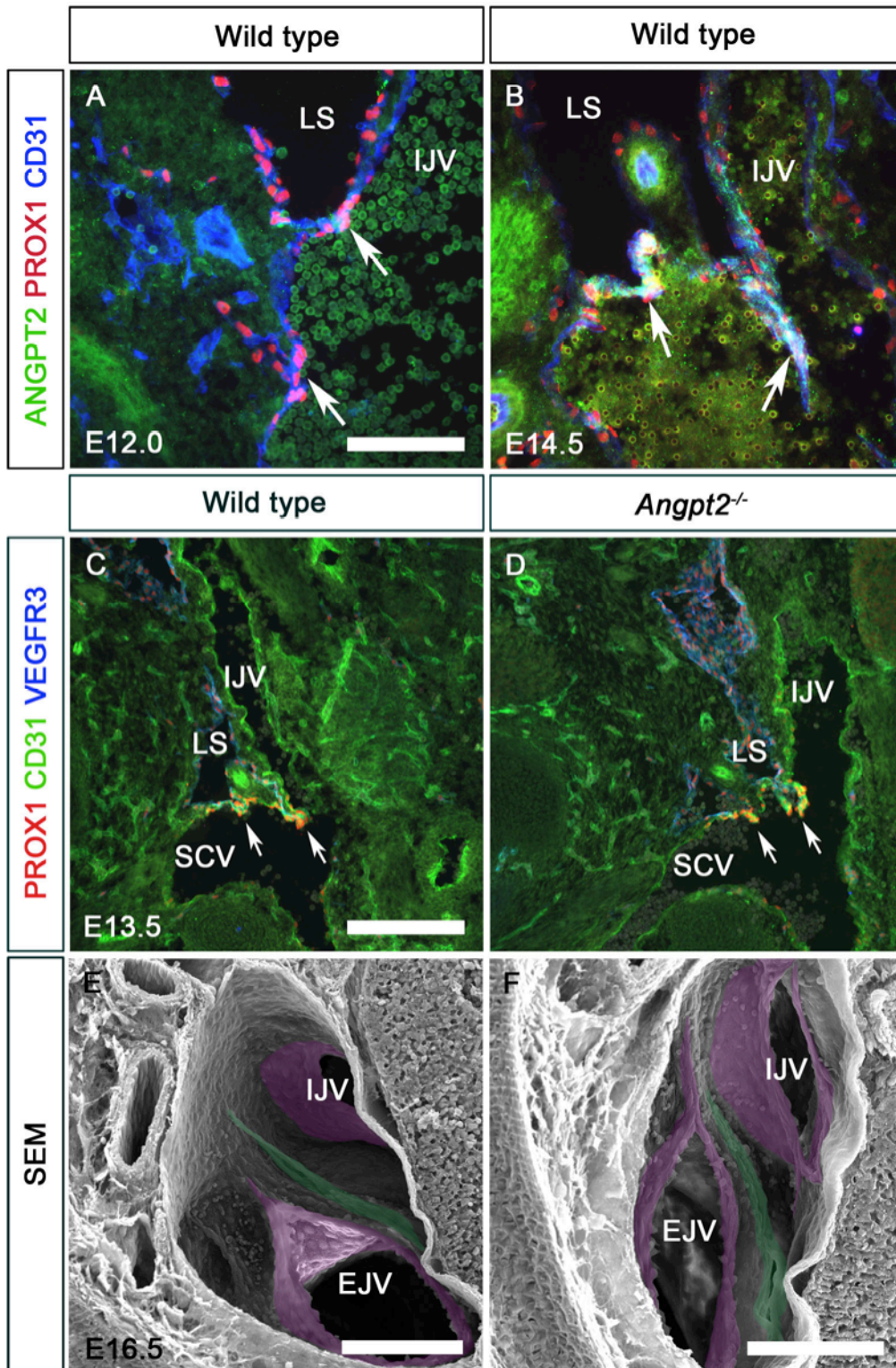
Figure 6

3
4

1   **Figure 6: Angiopoietin2 does not regulate LVV development.**
2
3   (A, B) ANGPT2 was not expressed in E12.0 LVV-ECs (A, arrows). However, it was expressed
4   in LVV-ECs at E14.5 (B, arrows).
5
6   (C, D) LVVs developed normally in E13.5 $Angpt2^{-/-}$ embryos (arrows).
7
8   (E, F) LVVs (green) and VVs (magenta) developed normally in E16.5 $Angpt2^{-/-}$ embryos.
9
10
11 Abbreviations: IJV, internal jugular vein; EJV, external jugular vein; LS, lymph sac.
12
13 Measuring bar: (A-B) 100 µm; (C-D) 200 µm; (E-F) 100 µm.
14
15 Statistics:(A-F) n= 3 embryos and 6 LVV complexes per genotype.
16

48

1
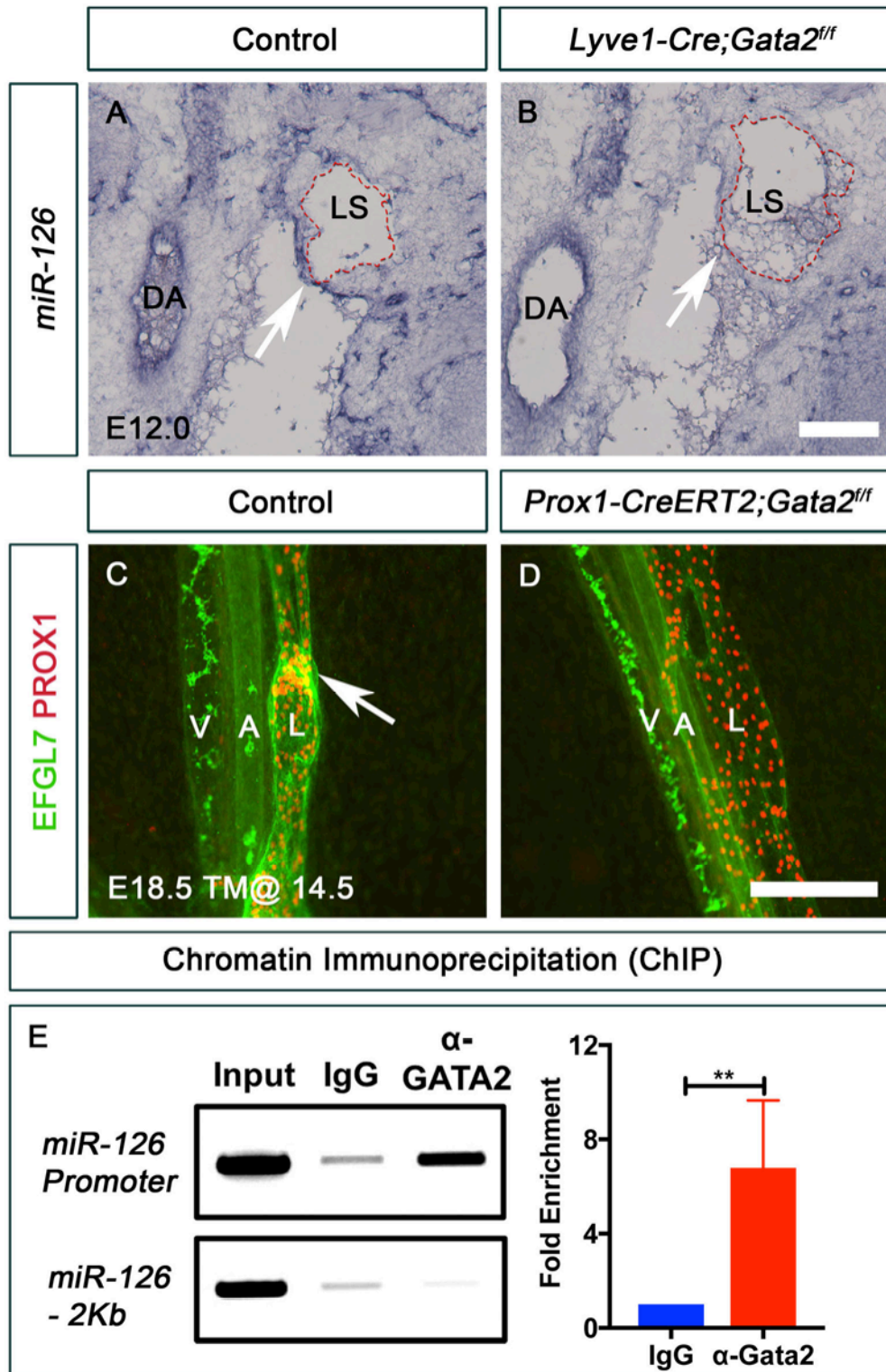
Figure 7

2
3

1 **Figure 7: *EGFL7/miR-126* is a target of GATA2.**

2

3 (A) miR-126 was expressed in the dorsal aorta (DA) and in the LVV-ECs (arrow) of control
4 embryos. (B) Expression of miR-126 was downregulated in the LVV-ECs (arrows) of E12.0
5 *Lyve1-Cre;Gata2$^{f/f}$* embryos. However, no obvious difference in miR-126 expression was
6 observed in the dorsal aorta (DA) of mutants.

7

8 (C) EGFL7 was expressed in the mesenteric arteries (A), veins (V) and lymphatic vessels (L)
9 of E18.5 control embryos. Strongest expression of EGFL7 was observed in LVs (arrow). (D)
10 Expression of EGFL7 was dramatically downregulated in the mesenteric lymphatic vessels of
11 mice lacking GATA2 in LECs. Also, notice the absence of LVs in the mutant.

12

13 (E) Chromatin immunoprecipitation (ChIP) revealed that GATA2 strongly associates with the
14 promoter element of the *EGFL7/miR-126* locus. The top lane of the gel picture was PCR
15 performed using primers flanking the GATA2 binding site. The lower lane was PCR performed
16 using primers for a non-specific site. The graph compares qPCR signals generated by primers
17 flanking the GATA2 binding site.

18

19 Measuring bar: (A, B) 250 µm; (C, D) 200 µm.

20

21 Statistics: (A, B) n= 3 embryos and 6 LVV complexes per genotype; (C-D) n=3 embryos per
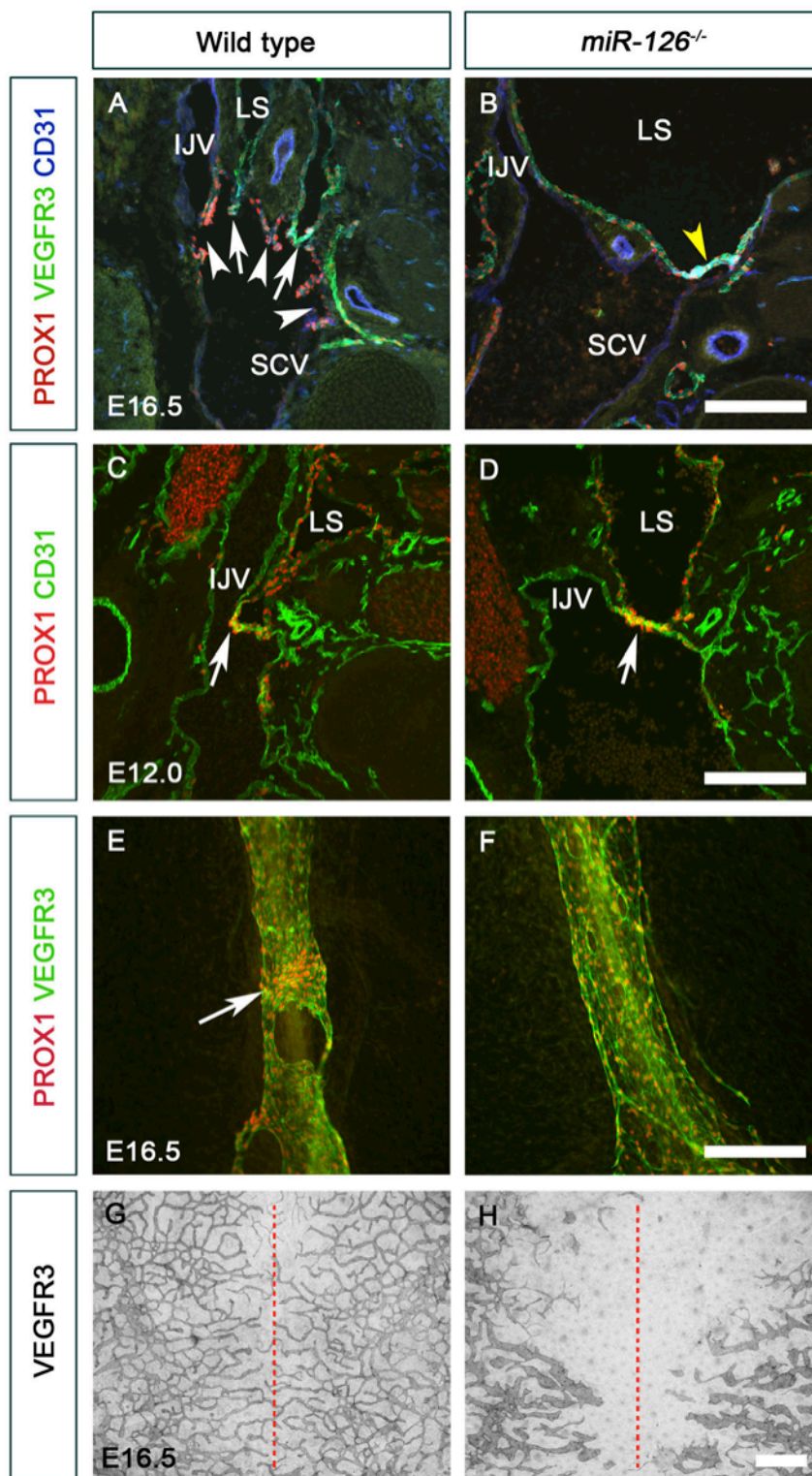22 genotype; (E) n=4. (**) p<0.01

23

24

25

26

27

28

50

1
2

Figure 8

3
4

1 **Figure 8: *miR-126$^{-/-}$* embryos are phenotypically similar to mice lacking *Gata2* in LECs.**
2
3 (A, B) LVVs (arrows) and VVs (arrowheads) were seen at the junction of internal jugular vein
4 (IJV) and subclavian vein (SCV) of E16.5 control (A) but not *miR-126$^{-/-}$* (B) embryos. A few
5 PROX1$^+$ cells were nevertheless seen at the interface of vein and lymph sacs (B, yellow
6 arrowhead).
7
8 (C, D) LVV-ECs were observed in both E12.0 control and *miR-126$^{-/-}$* littermates (arrows)
9 indicating that miR-126 is not necessary for the differentiation of LVV-ECs.
10
11 (E, F) LVs were observed in the mesenteric lymphatic vessels of E16.5 control embryos (E,
12 arrow). PROX1 expression was higher in the LVs compared to LECs. (F) LVs were absent and
13 PROX1 expression was homogeneous in the LECs of *miR-126$^{-/-}$* littermates.
14
15 (G, H) Lymphatic vessels in the dorsal skin had migrated from the lateral edges until the
16 midline (red dotted lines) in control (G) but not the *miR-126$^{-/-}$* (H) embryos. In addition, the
17 lymphatic vessels of the mutants were dilated with fewer branch points.
18
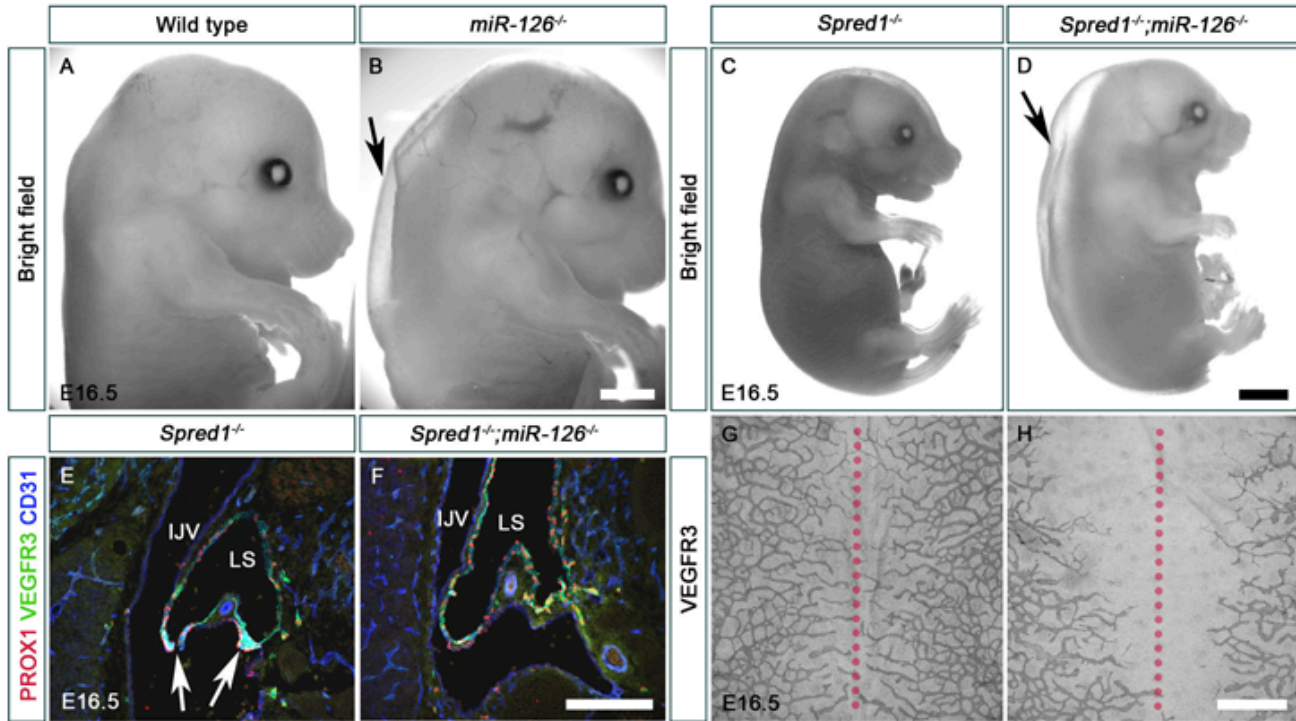19 Abbreviations: IJV, internal jugular vein; SCV, subclavian vein; LS, lymph sac.
20
21 Measuring bar: (A-F) 200 μm; (G, H) 1000 μm.
22
23 Statistics: (A-D) n= 3 embryos and 6 LVV complexes per genotype; (E-H) n= 3 embryos per
24 genotype.
25
26

Figure 9

Figure 9: Deletion of Spred1 does not rescue the lymphatic vascular phenotypes of *miR-126^{-/-}* embryos.

(A-D) Severe peripheral edema (arrows) was observed in E16.5 *miR-126^{-/-}* (B) and *miR-126^{-/-}* ; *Spred1^{-/-}* (D) embryos. (C) E16.5 *Spred1^{-/-}* embryos do not have obvious edema.

(E-H) E16.5 *Spred1^{-/-}* embryos had LVVs (E, arrows) and had normal lymphatic patterning (G). Deletion of *Spred1* does not rescue the absence of LVVs (F) or lymphatic vessel patterning defect (H) of *miR-126^{-/-}* embryos. The dotted lines indicate the midline of dorsal skin.
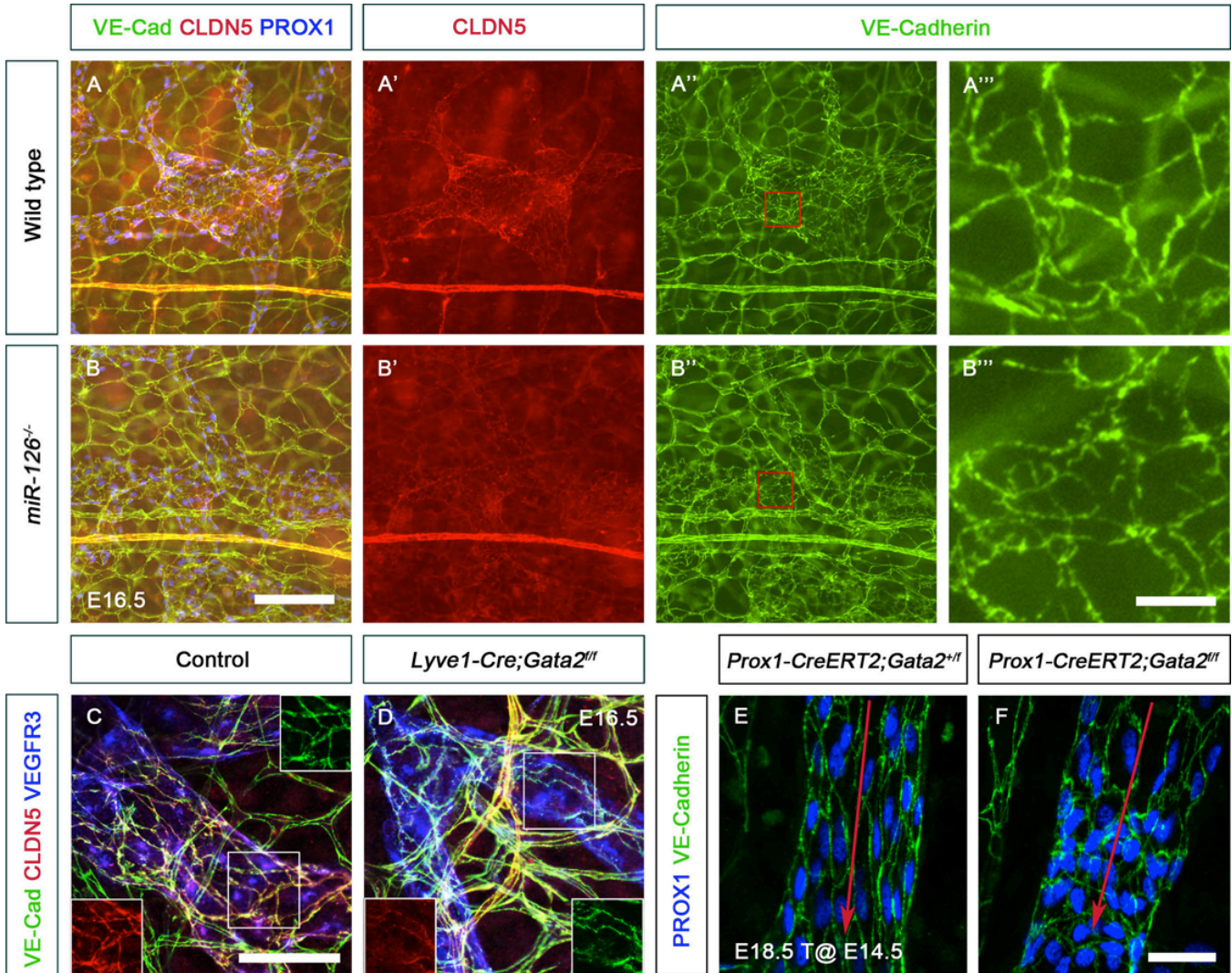
Abbreviations: IJV, internal jugular vein; LS, lymph sac.

Measuring bar: (A, B) 5000 µm; (C, D) 2500 µm; (E, F) 200 µm; (G, H) 1000 µm.

Statistics: (A-D, G-H) n= 3 embryos per genotype; (E, F) n=3 embryos, 6 LVV complexes per genotype.

Figure 10

1 **Figure 10: GATA2 and miR-126 are necessary for the expression of cell junction**
2 **molecules Claudin5 and VE-Cadherin.**
3
4 (A, B) Expression of tight junction molecule Claudin5 (CLDN5) was dramatically
5 downregulated in the dorsal skin lymphatic vessels of E16.5 *miR-126$^{-/-}$* embryos (B'). The
6 intensity of VE-Cadherin staining appeared to be not different between control (A'') and mutant
7 (B'') embryos. However, closer inspection revealed that VE-Cadherin was uniformly expressed
8 along the cell boundaries of control embryos (A'''), but was localized in a "zig zag" pattern in
9 mutant (B''') embryos.
10
11 (C, D) Claudin5 was downregulated (D, left inset) and VE-Cadherin had a defective localization
12 with gaps (D, right inset) in the dorsal skin lymphatic vessels of E16.5 *Lyve1-Cre;Gata2$^{f/f}$*
13 embryos.
14
15 (E) The LECs of mesenteric lymphatic vessels were elongated in the direction of lymph flow
16 (red arrow) in E18.5 control embryos. VE-Cadherin was uniformly expressed around the cell
17 boundaries of control LECs. In contrast, the LECs were misaligned and VE-Cadherin appeared
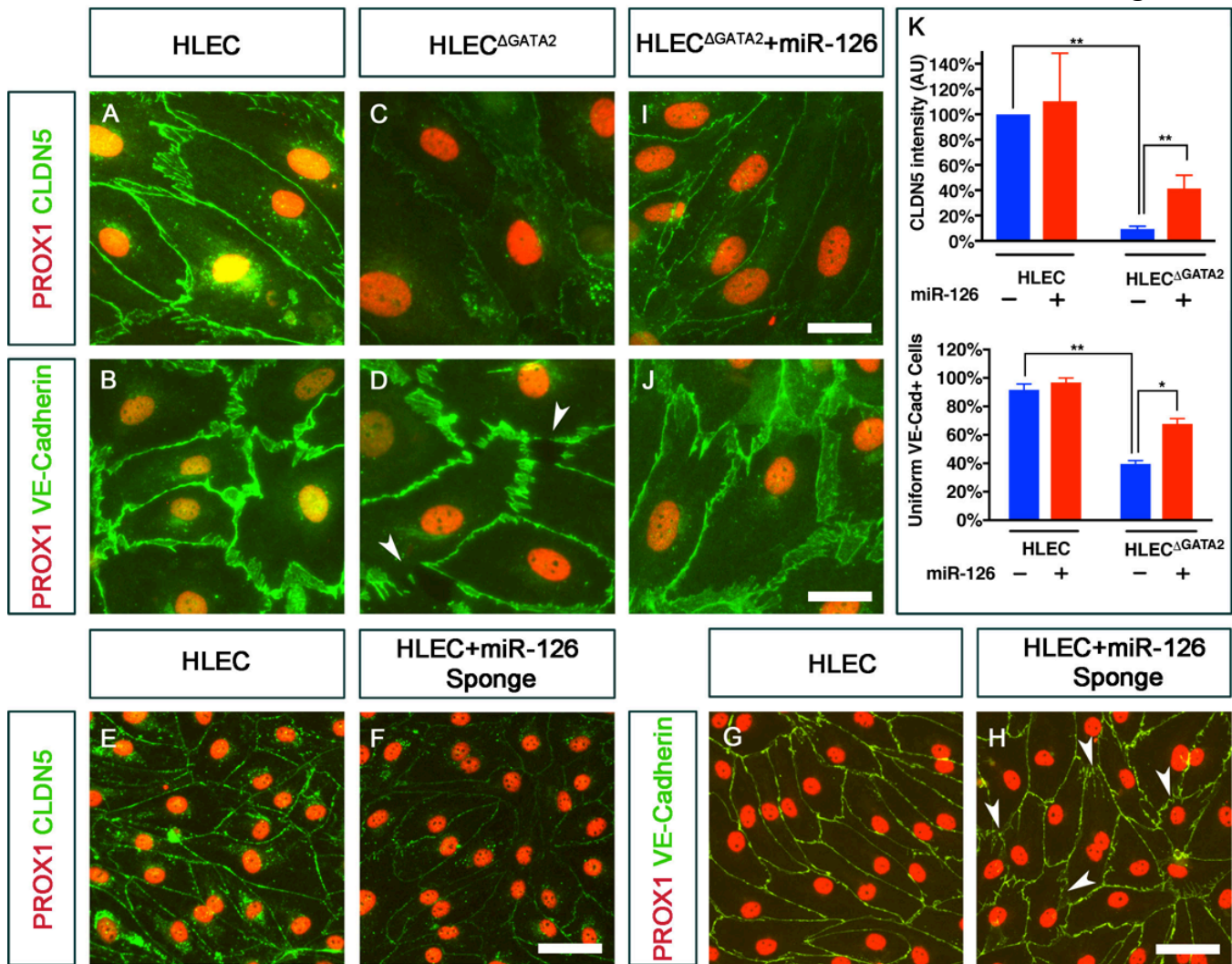18 to be mislocalized in embryos lacking GATA2 (F).
19
20 Measuring bar: (A, B) 200 μm; (A'''-B''') 25 μm; (C-F) 50 μm.
21
22 Statistics: n= 3 embryos per genotype.
23

Figure 11: GATA2 regulates the expression of Claudin5 and VE-Cadherin in HLECs in a miR-126 dependent manner.

(A) Claudin5 and (B) VE-Cadherin were uniformly expressed in the cell boundaries of control HLECs. In contrast, (C) Claudin5 was dramatically downregulated and (D) VE-Cadherin was expressed in a zig zag pattern (arrowheads) on the cell boundaries of HLEC$^{\Delta GATA2}$. (E-H) Expression of a miR-126 sponge using lentivirus strikingly downregulated Claudin5 (F) and caused disruptions in VE-Cadherin localization (H, arrowheads) in HLECs.

(I, J) Overexpression of miR-126 using lentiviral particles significantly rescued the expression of Claudin5 (I) and VE-Cadherin (J) expressions in HLEC$^{\Delta GATA2}$.
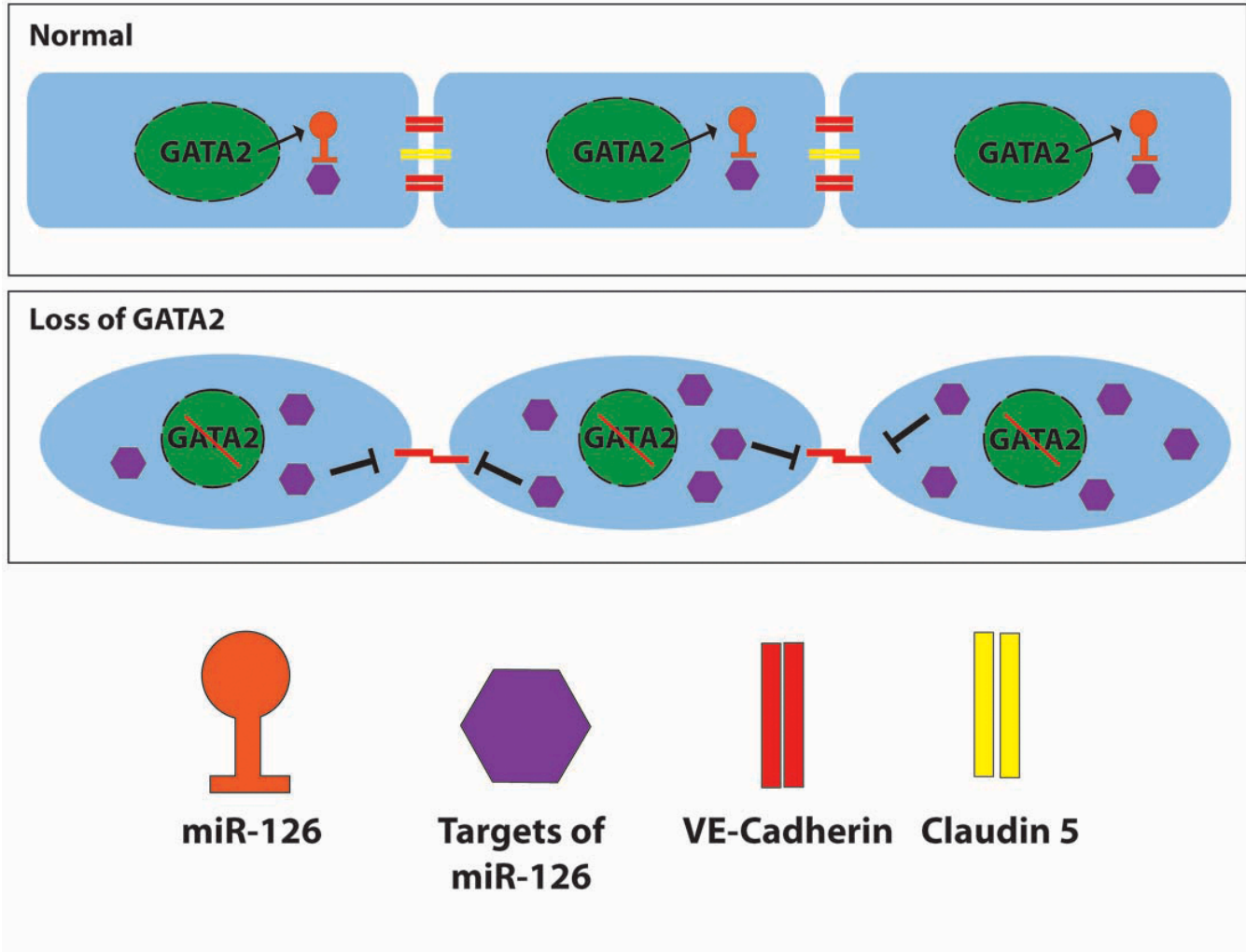
(K) The intensity of Claudin5 expression and the number of cells with uniform expression of VE-Cadherin were quantified and graphically shown in K.

Measuring bar: (A-F) 25 μm; (H-K) 50 μm.

Statistics: (A-D, E-H, I) n = 3; (J) n=2. (**) p<0.01; (*) p<0.05.

56

1   **Figure 12**

Figure 12: GATA2 regulates endothelial cell junctions through miR-126.

Under normal conditions GATA2 enhances the expression of miR-126 in the lymphatic vasculature. The targets of miR-126 are upregulated in the absence of GATA2, which results in the downregulation of Claudin5 and mislocalization of VE-Cadherin. Consequently, lymphatic vascular morphogenesis is defective due to abnormal shape of LECs and LVV-ECs.