Capillary malformation–arteriovenous malformation (CM-AVM) is a blood and lymphatic vessel (LV) disorder that is caused by inherited inactivating mutations of the RASA1 gene, which encodes p120 RasGAP (RASA1), a negative regulator of the Ras small GTP-binding protein. How RASA1 mutations lead to the LV leakage defects that occur in CM-AVM is not understood. Here, we report that disruption of the Rasa1 gene in adult mice resulted in loss of LV endothelial cells (LECs) specifically from the leaflets of intraluminal valves in collecting LVs. As a result, valves were unable to prevent fluid backflow and the vessels were ineffective pumps. Furthermore, disruption of Rasa1 in midgestation resulted in LEC apoptosis in developing LV valves and consequently failed LV valvulogenesis. Similar phenotypes were observed in induced RASA1-deficient adult mice and embryos expressing a catalytically inactive RASA1R780Q mutation. Thus, RASA1 catalytic activity is essential for the function and development of LV valves. These data provide a partial explanation for LV leakage defects and potentially other LV abnormalities observed in CM-AVM.

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RASA1 regulates the function of lymphatic vessel valves in mice

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Capillary malformation–arteriovenous malformation (CM-AVM) is a blood and lymphatic vessel (LV) disorder that is caused by inherited inactivating mutations of the RASA1 gene, which encodes p120 RasGAP (RASA1), a negative regulator of the Ras small GTP-binding protein. How RASA1 mutations lead to the LV leakage defects that occur in CM-AVM is not understood. Here, we report that disruption of the Rasa1 gene in adult mice resulted in loss of LV endothelial cells (LECs) specifically from the leaflets of intraluminal valves in collecting LVs. As a result, valves were unable to prevent fluid backflow and the vessels were ineffective pumps. Furthermore, disruption of Rasa1 in midgestation resulted in LEC apoptosis in developing LV valves and consequently failed LV valvulogenesis. Similar phenotypes were observed in induced RASA1-deficient adult mice and embryos expressing a catalytically inactive RASA1R780Q mutation. Thus, RASA1 catalytic activity is essential for the function and development of LV valves. These data provide a partial explanation for LV leakage defects and potentially other LV abnormalities observed in CM-AVM.

Introduction

Capillary malformation–arteriovenous malformation (CM-AVM) is an autosomal dominant vascular disorder that affects at least 1:100,000 individuals in Northern European populations (1–3). The pathognomonic feature of CM-AVM is the presence of 1 or more small randomly distributed cutaneous CMs. In one-third of patients, there are additional fast flow blood vascular (BV) lesions, such as AVM, arteriovenous fistulas (AVF), or Parkes Weber syndrome, in which AVF and CM are associated with overgrowth of a single limb. Lymphatic vessel (LV) disorders have also been described in some CM-AVM patients. These include chylothorax and chylos ascites (accumulation of lymph in the pleural and peritoneal cavities, respectively), lymphedema, LV malformation, and hyperplasia (2–6). In 70% of CM-AVM patients, inherited inactivating mutations of the RASA1 gene are responsible for disease development. RASA1 is a Ras GTPase–activating protein (RasGAP) that accelerates conversion of the inner membrane–tethered Ras protein from an active GTP-bound state back to an inactive GDP-bound state during growth factor receptor signal transduction (7). In addition, RASA1 has been reported to perform distinct functions in intracellular signal transduction that are independent of its ability to promote Ras hydrolysis of GTP (7, 8).

Germline RASA1 mutations in CM-AVM span the entire gene, and the vast majority are nonsense mutations, splice site substitutions, or insertions or deletions that are all predicted to result in rapid RNA transcript degradation as a consequence of nonsense-mediated RNA decay (9). In affected families with RASA1 mutations, 95% of individuals that inherit a mutated RASA1 gene develop vascular lesions, attesting to the high penetrance of these mutations (1–3). It has been proposed that development of vascular lesions in patients with germline RASA1 mutations additionally requires somatic mutation of the inherited normal RASA1 allele, resulting in the complete loss of RASA1 protein in affected cells (2, 3). In support of this hypothesis, a RASA1 somatic mutation was recently identified in a CM of a CM-AVM patient with an inherited RASA1 mutation (5).

Consistent with the vascular anomalies in humans with RASA1 mutations, homozygous Rasa1-null mice show defects in vascular development and die in midgestation (10). In the yolk sac, newly formed BV endothelial cells (BECs) fail to organize into a vascular network that supplies blood to the embryo. In the embryo proper, the developing dorsal aorta is irregular, with abnormal projecting arteries and local hemorrhage, and distension of the pericardial sac is observed. The same phenotype is observed in mice in which the Rasa1 gene is disrupted specifically in BECs, showing that the BV abnormalities that occur in global Rasa1-null mice are intrinsic to BECs (11). Furthermore, the same BV abnormalities occur in homozygous Rasa1R780Q mice, which express a mutant RASA1 protein that is incapacitated in its ability to negatively regulate Ras specifically (12). Thus, BV abnormalities in Rasa1 null mice result from loss of RASA1-mediated control of Ras signaling rather than loss of noncatalytic functions of RASA1.

Induced disruption of the Rasa1 gene in adult mice does not result in spontaneous BV abnormalities, but instead causes a striking LV defect that parallels the LV abnormalities seen in some CM-AVM patients (11). Starting at 1 to 2 months after gene disruption, mice develop chylothorax and chylos ascites and succumb to the former. Mice also develop extensive LV hyperplasia, particularly at the end stage of disease. These lymphatic phenotypes...
contractile vessels (13, 14). LV valvulogenesis is initiated in late gestation and proceeds through 4 distinct steps (15–20). In the first step, oscillatory shear stress contributes to the formation of rings of valve-forming cells with increased expression of prospe-ro homeobox 1 (PROX1), forkhead box C2 (FOXC2), and GATA binding protein 2 (GATA2) transcription factors, which drive subsequent steps of valve formation. In the second step, known as condensation, the rings compress and valve-forming cells orient 90° toward the vessel lumen. This is accompanied by the nuclear mobilization of the NFATc1 transcription factor that is dependent upon the GAP junction protein connexin 37 (CX37). In the third step, valve-forming cells protrude into the vessel lumen, elongate in the direction of lymph flow, and produce extracellular matrix (ECM) proteins (e.g., laminin α5, fibronectin-EIIIA, collagen IV, and EMILIN1), which form the core of the valve leaflet. Elongation is mediated by integrin α-9, which anchors the valve-forming cells to the ECM. In the fourth step, additional ECM deposition results in the thickening of leaflets and valve maturation. Mice that were also observed in mice in which the Rasa1 gene was disrupted specifically within LV endothelial cells (LECs), showing that they result from loss of RASA1 within this cell type (11). Development of LV hyperplasia was attributed to dysregulated signaling through VEGFR3 in LECs. However, whether any of the LV abnormalities in these mice result from dysregulated Ras activation or perturbations in other signaling pathways was not addressed. In addition, the basis of LV leakage defects in induced RASA1-deficient mice was not determined. These are important questions, since answers are likely to provide insights into the pathogenesis of LV abnormalities in patients with CM-AVM as well as suggest possible means of treatment.

In previous near-infrared fluorescence lymphatic imaging (NIRFLI) studies of live induced RASA1-deficient mice and humans with RASA1 mutations, we noticed back-flow of dye at injection sites suggestive of an LV valve defect that could account for leakage (4, 11). Collecting LVs contain intraluminal semilunar valves that are essential for propulsive lymph flow in these contractile vessels (13, 14). LV valvulogenesis is initiated in late gestation and proceeds through 4 distinct steps (15–20). In the first step, oscillatory shear stress contributes to the formation of rings of valve-forming cells with increased expression of prospe-ro homeobox 1 (PROX1), forkhead box C2 (FOXC2), and GATA binding protein 2 (GATA2) transcription factors, which drive subsequent steps of valve formation. In the second step, known as condensation, the rings compress and valve-forming cells orient 90° toward the vessel lumen. This is accompanied by the nuclear mobilization of the NFATc1 transcription factor that is dependent upon the GAP junction protein connexin 37 (CX37). In the third step, valve-forming cells protrude into the vessel lumen, elongate in the direction of lymph flow, and produce extracellular matrix (ECM) proteins (e.g., laminin α5, fibronectin-EIIIA, collagen IV, and EMILIN1), which form the core of the valve leaflet. Elongation is mediated by integrin α-9, which anchors the valve-forming cells to the ECM. In the fourth step, additional ECM deposition results in the thickening of leaflets and valve maturation. Mice that

Figure 1. Impaired pumping function of induced RASA1-deficient LVs. (A) Examples of traces from pump function assays performed with LVs from littermate Rasa1flo/flo and Rasa1flo/flo Ubert2cre mice treated with tamoxifen 9 weeks beforehand. Tests were performed as indicated for contraction assays in Supplemental Figure 1 except that P

\[ \text{out} \] (cm H2O) was kept at 0.5 cm H2O as P

\[ \text{in} \] was increased from 0.5 to 10 cm H2O in ramp-wise fashion. The position of the downstream valve as open or closed is shown at top. In traces at left, asterisks indicate the point of pump failure where the downstream valve locks, at least temporarily, into an open position. Traces at right show magnified regions from left traces near the beginning of the P

\[ \text{out} \] ramps. Drop-down dotted lines are to illustrate at which point in the vessel contraction cycle valves open. Note valve opening during systole in the Rasa1flo/flo vessel and during diastole in the Rasa1flo/flo Ubert2cre vessel. (B) Shown is the mean pump limit plus SEM for vessels from littermate Rasa1flo/flo (n = 4) and Rasa1flo/flo Ubert2cre mice (n = 5) in pump-function tests. The pump limit is defined as the adverse pressure (P

\[ \text{out} \] – P

\[ \text{in} \] ) at pump failure. **P < 0.0025, Student’s 2-sample t test.
In this study, we used an inducible RASA1-deficient mouse model to examine whether RASA1 is necessary for the normal functioning of adult LV valves. In addition, we used an inducible Rasa1R780Q mouse model to examine whether lymphatic phenotypes that develop in induced RASA1-deficient mice, including potential valve dysfunction, result from loss of RASA1 catalytic activity directed toward Ras or to loss of a noncatalytic function of RASA1. Whether RASA1 catalytic activity is required for lymphatic vasculogenesis was also examined in these studies. We report an essential function for RASA1 in valve maintenance and development that is partly dependent or completely dependent upon

are deficient in genes that are expressed in LV valves illustrate the importance of the respective proteins at different steps of valve formation. For example, FOXC2-deficient mice fail to develop valves at all (21). In contrast, CX37-deficient, CNB1-deficient (CNB1, a calcineurin subunit, is required for NFAT signaling), and integrin α-9–deficient mice develop valves that arrest at stages 1, 2, and 3, respectively (15, 16, 22). Often, the same genes involved in the development of LV valves are also required for valve maintenance in adults, e.g., FOXC2 and GATA2 (20, 23). In contrast, other genes appear to be required for valve development but not maintenance, e.g., integrin α-9 (15, 24).

Figure 2. Valves in induced RASA1-deficient LVs fail to close in valve-closure tests. (A) Popliteal LVs, trimmed to contain a single valve, were cannulated with pipettes to allow control of upstream (P_in) and downstream (P_out) pressure. Assays were conducted in calcium-free medium to prevent vessel contraction. P_in was held constant, and P_out was increased until valve closure, assessed by a drop in upstream pressure as measured with the use of a servo-null micropipette (PsIn) and by a decrease in vessel diameter. The position of the diameter-tracking window is shown. The amount of adverse pressure (P_out − P_in) required for valve closure over a range of P_in and initial vessel diameters was determined. (B) Examples of traces from valve-closure tests performed with LVs from littermate Rasa1fl/− and Rasa1fl/− Ubert2cre mice administered tamoxifen 9 weeks previously. For control Rasa1fl/− LVs, the point of valve closure is indicated with an asterisk. Adverse pressure required for valve closure is represented by the difference between the dotted and P_in lines. Note that increased amounts of adverse pressure are required for valve closure at increased P_in and initial vessel diameter. Note also apparent failed valve closure in the Rasa1fl/− Ubert2cre LVs up to maximum tested adverse pressures of 30 cm H2O at either tested P_in. (C) Plots of adverse pressure required for valve closure at different vessel diameters (D) represented as D/D_max, where D_max is defined as vessel diameter at a maximum tested P_in of 10 cm H2O. Individual valves from littermate Rasa1fl/− (n = 4) and Rasa1fl/− Ubert2cre (n = 5) mice treated with tamoxifen 9 weeks previously are represented by the same colored symbols. (D) Plots of adverse pressure required for valve closure over a range of D/D_max of LVs from littermate Rasa1fl/− (n = 6) and Rasa1fl/− Ubert2cre (n = 6) mice treated with tamoxifen 1 week previously.
valve 1 and downstream (P_{out}) of valve 2. Pin and P_{out} were changed in parallel, and the amplitude and frequency of contractions were determined by measurement of vessel diameter between the 2 valves. In Rasa1^{fl/fl} control vessels, as intraluminal pressure was increased, the frequency of contractions increased and contraction amplitude decreased, as expected (Supplemental Figure 1, B and C). Moreover, in vessels from Rasa1^{fl/fl} Ubert2^{cre} mice (treated with tamoxifen 9 weeks previously), similar changes in contraction frequency and amplitude were noted in response to increased intraluminal pressure (Supplemental Figure 1, B and C). Ejection fraction and fractional pump flow rate were also comparable between the 2 groups of mice (Supplemental Figure 1C). These findings indicate that the contractile function of collecting LVs is not affected by the loss of RASA1.

We next compared the ability of control and RASA1-deficient LVs to pump fluid against an adverse pressure gradient. Efficient pumping requires normal valve function as well as robust muscle contraction (25). Experiments were conducted similarly to contraction assays, except that only P_{out} was increased while Pin was held at a constant low level of 0.5 cm H_{2}O. Video recordings of the downstream (P_{out}) of valve 2. Pin and P_{out} were changed in parallel, and the amplitude and frequency of contractions were determined by measurement of vessel diameter between the 2 valves. In Rasa1^{fl/fl} control vessels, as intraluminal pressure was increased, the frequency of contractions increased and contraction amplitude decreased, as expected (Supplemental Figure 1, B and C). Moreover, in vessels from Rasa1^{fl/fl} Ubert2^{cre} mice (treated with tamoxifen 9 weeks previously), similar changes in contraction frequency and amplitude were noted in response to increased intraluminal pressure (Supplemental Figure 1, B and C). Ejection fraction and fractional pump flow rate were also comparable between the 2 groups of mice (Supplemental Figure 1C). These findings indicate that the contractile function of collecting LVs is not affected by the loss of RASA1.

RASA1 catalytic activity in these respective roles. In valve development, we further show that a role for RASA1 is explained by an essential function in the survival of LECs in valve leaflets.

**Results**

***Induced RASA1-deficient LVs are ineffective as pumps.*** The majority of assays of LV function ex vivo were performed upon afferent popliteal collecting LVs. To induce loss of RASA1 protein in vessels, we used mice carrying conditional floxed Rasa1 alleles (Rasa1^{fl/fl}) and ubiquitin-3 promoter-driven, tamoxifen-inducible Cre (Ubb3-CreER<T>), referred to here as Ubert2^{cre}. Rasa1^{fl/fl} and Rasa1^{fl/fl} Ubert2^{cre} mice were administered tamoxifen at 2 months of age. Littermate Rasa1^{fl/fl} mice were also administered tamoxifen at the same time and served as controls. We first examined contractile activity of vessels (Supplemental Figure 1A). In Rasa1^{fl/fl} Ubert2^{cre} mice, mice administered tamoxifen at 2 months of age. Littermate Rasa1^{fl/fl} mice were also administered tamoxifen at the same time and served as controls. We first examined contractile activity of vessels (Supplemental Figure 1A; supplemental material available online with this article; https://doi.org/10.1172/JCI89607DS1). These assays were conducted in medium containing calcium to permit spontaneous vessel contraction. Trimmed vessels that contained 2 valves were cannulated at open ends to allow manipulation of intraluminal pressure upstream (P_{in}) of valve 1 and downstream (P_{out}) of valve 2. Pin and P_{out} were changed in parallel, and the amplitude and frequency of contractions were determined by measurement of vessel diameter between the 2 valves. In Rasa1^{fl/fl} control vessels, as intraluminal pressure was increased, the frequency of contractions increased and contraction amplitude decreased, as expected (Supplemental Figure 1, B and C). Moreover, in vessels from Rasa1^{fl/fl} Ubert2^{cre} mice (treated with tamoxifen 9 weeks previously), similar changes in contraction frequency and amplitude were noted in response to increased intraluminal pressure (Supplemental Figure 1, B and C). Ejection fraction and fractional pump flow rate were also comparable between the 2 groups of mice (Supplemental Figure 1C). These findings indicate that the contractile function of collecting LVs is not affected by the loss of RASA1.

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stream valve permitted a determination of the pump limit, defined as the value of adverse pressure (P_{out} – P_{in}) at which the downstream valve locks into a closed or open position (Figure 1, A and B). LVs from control Rasa1^{fl/fl} mice behaved as expected in these assays and were able to effectively pump fluid against modest adverse pressure gradients (2–4.8 cm H₂O). In contrast, LVs from Rasa1^{fl/fl} Prox1^ert2cre mice treated with tamoxifen 9 weeks previously were ineffective as pumps. Whereas in control vessels, the downstream valve was mostly in a closed position throughout the P_{out} ramp, in the RASA1-deficient vessels, the downstream valve was mostly in an open position (Figure 1A). Correlation of vessel diameter with valve position provided further information upon the basis of the impaired pumping function of the RASA1-deficient vessels. In control vessels, valves opened during systole and closed during diastole under conditions of adverse pressure up to the pump limit. In contrast, in RASA1-deficient vessels, the downstream valve reopened inappropriately during the diastolic phase of the vessel contraction cycle (Figure 1A). Since contraction frequency and amplitude were not altered (Supplemental Figure 1), these findings suggested that the RASA1-deficient vessels have a primary valve defect that would account for the impaired pumping function.

Induced RASA1-deficient LV valves fail to close properly in response to adverse intraluminal pressure. To examine valve function directly, we performed valve-closure tests upon control and RASA1-deficient vessels. For these experiments, vessels from tamoxifen-treated Rasa1^{fl/fl} and Rasa1^{fl/fl} Prox1^ert2cre mice were trimmed.

Figure 4. Specific induced loss of RASA1 within LECs results in dysfunction of popliteal and mesenteric LV valves. LV valve function tests were conducted with popliteal LV valves from Rasa1^{fl/fl} Prox1^ert2cre mice administered tamoxifen 11 weeks previously (A–C) (n = 7) or mesenteric LV valves from littermate Rasa1^{fl/fl} and Rasa1^{fl/fl} Prox1^ert2cre mice administered tamoxifen 20 weeks previously (D–F) (n = 8 and 10, respectively). (A and D) Valve-closure tests. Graphs show adverse pressure required for valve closure versus D/D_{max}. Each valve is represented by a different colored symbol. (B and E) Graphs show Psn as a function of P_{out} in low-pressure back-leak tests. (C and F) Graphs show Psn as a function of P_{out} in high-pressure back-leak tests. The same colored symbols are used for the same valves in graphs A–C and D–F. See also the following: Supplemental Video 3, which shows a low-pressure back-leak test of a Rasa1^{fl/fl} Prox1^ert2cre popliteal LV valve that failed to close and leaked (gray circles in A–C); Supplemental Video 4, which shows a high-pressure back-leak test of a control Rasa1^{fl/fl} popliteal LV valve; Supplemental Video 5, which shows a high-pressure back-leak test of a Rasa1^{fl/fl} Prox1^ert2cre popliteal LV valve that closed at a P_{in} of 50 cm H₂O and prevented further back-leak thereafter (red triangles in A–C); Supplemental Video 6, which shows a low-pressure back-leak test of a Rasa1^{fl/fl} Prox1^ert2cre mesenteric LV valve; Supplemental Video 7, which shows a low-pressure back-leak test of a Rasa1^{fl/fl} Prox1^ert2cre mesenteric LV valve (blue circles in D–F).
Figure 5. LV valve leaflet atrophy associated with reduced numbers of leaflet PROX1+ LECs in induced RASA1-deficient mice. All analyses were performed with popliteal LV valves. (A) Still image of popliteal LV valve from a back-leak experiment indicating parameters measured for B–D. (B) Mean vessel diameter ± SEM at different upstream pressures (P_in) of LVs from Rasa1<sup>fl/fl</sup> (n = 4) and Rasa1<sup>fl/fl</sup> Ubert2cre (n = 7) mice treated with tamoxifen 9 weeks previously. (C) Mean sinus diameter ± SEM at P_out 10 cm H<sub>2</sub>O of LVs from littermate Rasa1<sup>fl/fl</sup> and Rasa1<sup>fl/fl</sup> Ubert2cre mice treated with tamoxifen 1, 9, and 16 weeks previously (n = 6 of each genotype at 1 and 16 weeks; n = 3 Rasa1<sup>fl/fl</sup> and n = 7 Rasa1<sup>fl/fl</sup> Ubert2cre mice at 9 weeks after tamoxifen treatment). (D) Mean leaflet length ± SEM of LVs from littermate Rasa1<sup>fl/fl</sup> and Rasa1<sup>fl/fl</sup> Ubert2cre mice treated with tamoxifen 1, 9, and 16 weeks previously (n = 12 of each genotype at 1 and 16 weeks; n = 6 Rasa1<sup>fl/fl</sup> and n = 13 Rasa1<sup>fl/fl</sup> Ubert2cre at 9 weeks after tamoxifen treatment). (E) Confocal microscopy images of popliteal LVs from littermate Rasa1<sup>fl/fl</sup> and Rasa1<sup>fl/fl</sup> Ubert2cre mice administered tamoxifen 5 weeks beforehand. Vessels were stained with integrin α-9 and PROX1 antibodies to highlight valve leaflets. Larger images at left show whole-valve regions. Smaller images at right show valve leaflets only from the same valves. One valve leaflet image is marked to indicate maximum leaflet depth, and outer perimeter is shown in F. (F) Graphs show maximum leaflet depth, leaflet outer perimeter, and number of PROX1+ cells per leaflet for different LV valve leaflets from Rasa1<sup>fl/fl</sup> and Rasa1<sup>fl/fl</sup> Ubert2cre mice administered tamoxifen 5 weeks beforehand. Mean ± SEM are indicated. *P < 0.05; ****P < 0.0001, Student’s 2-sample t test.
further so that they contained a single valve before they were recannulated at both ends (Figure 2A). Assays were conducted in calcium-free media to prevent lymphatic muscle contraction. P_out was increased, and the value of P_out required to close valves was determined at different values of P_in and, consequently, upstream vessel diameters. The point at which valves closed was assessed with the use of a servo-null micropipette (Psn) inserted through the vessel wall into the upstream vessel lumen to detect decreases in upstream intraluminal pressure that would be expected upon valve closure. In addition, upstream vessel diameter was recorded to provide confirmation of valve closure.

Valves from control Rasa1fl/fl mice behaved as expected in closure tests. As reported previously for rat LV valves, the amount of adverse pressure (P_out – P_in) required to close valves increased with increased upstream vessel diameter (Figure 2, B and C). However, even at maximum diameter, all control valves closed with moderate adverse pressures that did not exceed 15 cm H2O. In contrast, all examined valves from Rasa1fl/fl Ubert2cre mice treated with tamoxifen 9 weeks earlier failed to close at any tested upstream vessel diameter up to maximum tested adverse pressures in the range of 20–30 cm H2O (Figure 2, B and C). Thus, RASA1-deficient LV valves are unable to close properly in response to adverse intraluminal pressure.

To determine how soon the valve closure defect manifests after loss of RASA1, we also examined vessels from Rasa1fl/fl and Rasa1fl/fl Ubert2cre mice that had been treated with tamoxifen 1 week earlier (Figure 2D). All examined valves from the Rasa1fl/fl mice behaved normally. In contrast, abnormal valve closure was evident in 4 out of 6 tested valves from Rasa1fl/fl Ubert2cre mice. One valve failed to close at any initial vessel diameter, 2 required abnormally high adverse pressures for closure at small vessel diameters, and 1 required higher than normal adverse pressures for closure at large vessel diameters. The other 2 examined valves behaved similarly to controls (Figure 2D). Therefore, abnormalities of valve closure can be detected at an early time point after RASA1 loss.

Back-leak across induced RASA1-deficient LV valves. We attempted to measure the extent to which closed valves in RASA1-deficient LVs could resist back-leak of elevated intraluminal pressure downstream of the valve. As for valve-closure tests, back-leak assays were performed with vessels that contained a single valve in calcium-free medium. In low-pressure back-leak tests, P_in was kept at a constant low level of 0.5 cm H2O and P_out was elevated over a physiological range from 0.5 to 10 cm H2O. Psn recordings provided an indication of the extent of back-leak (Figure 3A). As in closure tests, valves from control Rasa1fl/fl mice closed spontaneously in response to small amounts of adverse pressure and, furthermore, resisted transfer of pressure to the upstream vessel lumen throughout the entire P_out range (Figure 3, A, C, and E).

Since RASA1-deficient valves failed to close properly in valve-closure tests, initial valve closure in low-pressure back-leak tests was encouraged by tapping of the vessel line to introduce spikes of intraluminal pressure (Figure 3A). Nonetheless, valves from Rasa1fl/fl Ubert2cre mice treated with tamoxifen 9 weeks previously failed to prevent transfer of pressure to the upstream lumen throughout the P_out range (Figure 3, A and C). Back-leak across valves from Rasa1fl/fl Ubert2cre mice in low-pressure back-leak tests was apparent as soon as 1 week for some valves (see later) and persisted until at least 16 weeks after tamoxifen treatment (Figure 3E).

In high-pressure back-leak tests, we measured valve back-leak under conditions of supraphysiological downstream pressure up to 100 cm H2O (Figure 3B). All tested valves from Rasa1fl/fl mice were able to resist increased downstream pressure throughout the P_out range in these tests (Figure 3, B and D). In contrast, valves from Rasa1fl/fl Ubert2cre mice treated with tamoxifen 9 weeks earlier were unable to resist these pressure increases (Figure 3, B, D, and F). Subsequent analysis of video recordings of valves in low- and high-pressure back-leak tests revealed that valves from Rasa1fl/fl Ubert2cre mice failed to close properly in both types of tests despite the line tapping or very high downstream pressures, respectively (Supplemental Videos 1 and 2 and data not shown). Failure of valve closure, therefore, accounts for the back-leak.

Specific induced disruption of the Rasa1 gene in LECs results in impaired LV valve function. To determine whether loss of RASA1 in LECs was sufficient for the development of LV valve dysfunction, we examined popliteal LVs from Rasa1fl/fl Prox1ert2cre, which express a lymphatic endothelial cell–specific tamoxifen-inducible Cre. Eleven weeks after tamoxifen administration to adult mice, popliteal LVs were tested in valve closure assays and low- and high-pressure back-leak tests (Figure 4, A–C). In valve-closure tests, 4 out of 7 tested valves failed to close at any tested vessel diameter and adverse pressure and 1 closed only at low vessel diameter with high adverse pressure (Figure 4A). Two other valves closed normally (Figure 4A). All of the 5 valves that were unable to close properly in closure tests were unable to resist back-leak in low- and high-pressure back-leak tests (Figure 4, B and C). Video analysis of valves in low-pressure back-leak tests confirmed that, for each of these 5 valves, valve closure never occurred, thereby accounting for back-leak (Supplemental Video 3). However, for 2 of these valves, valve closure did occur at a P_out of 50 cm H2O in high-pressure back-leak tests, which prevented further elevations in upstream pressure (Figure 4C and Supplemental Videos 4 and 5). Both of the other 2 valves that closed normally in valve-closure tests also closed normally in low- and high-pressure back-leak tests and resisted back-leak throughout the P_out ramps in those tests (Figure 4, B and C). In summary, the majority of valves from tamoxifen-treated Rasa1fl/fl Prox1ert2cre mice are dysfunctional, indicating that the valve defects observed in tamoxifen-treated Rasa1fl/fl Ubert2cre mice result from loss of RASA1 specifically in LECs. That some valves from tamoxifen-treated Rasa1fl/fl Prox1ert2cre mice function normally is most likely explained by less efficient Rasa1 gene disruption using the Prox1ert2cre driver compared with the Ubert2cre driver as we reported previously (11).

Induced loss of RASA1 in LECs results in defects in the function of mesenteric as well as popliteal LV valves. We examined whether specific induced disruption of RASA1 in LECs results in valve dysfunction in other types of LVs. For this purpose, we tested mesenteric LVs from Rasa1fl/fl and Rasa1fl/fl Prox1ert2cre mice treated with tamoxifen 20 weeks previously (Figure 4, D–F). In valve-closure tests, 4 out of 10 tested mesenteric LV valves from Rasa1fl/fl Prox1ert2cre mice failed to close properly, whereas all 8 tested mesenteric LV valves from Rasa1fl/fl mice behaved normally in these tests (Figure 4D). In back-leak tests, the same 4 dysfunctional valves from Rasa1fl/fl Prox1ert2cre mice were also unable to resist back-leak. In addition, a fifth valve from Rasa1fl/fl Prox1ert2cre mice, which behaved normally in valve-closure tests, showed back-leak in back-leak tests (Figure
4, D–F). In contrast, all 8 tested mesenteric LV valves from Rasa1β/β mice were able to resist back-leak (Figure 4, D–F). Video analysis of low-pressure back-leak tests confirmed that, for dysfunctional mesenteric LV valves from Rasa1β/β Prox1ERT2cre mice, valve closure never occurred (Supplemental Videos 6 and 7). Thus, specific loss of RASA1 in LECs results in dysfunctional mesenteric LV valves as well as popliteal LV valves.

Reduced length and cellularity of valve leaflets in LVs of induced RASA1-deficient mice. Increased vessel wall or sinus diameter or decreased valve leaflet length could each contribute to impaired LV valve closure. Therefore, we examined each of these parameters in popliteal LVs from tamoxifen-treated Rasa1β/β Rasa1fl/fl and Rasa1β/β Ubert2cre mice (Figure 5A). At 9 weeks after tamoxifen treatment, no difference in upstream vessel diameter was noted between the 2 types of mice over a range of \( P_\mathrm{in} \) pressures from 0.5 to 10 cm H\(_2\)O (Figure 5B). Likewise, at 1 and 9 weeks after tamoxifen treatment, no difference in sinus diameter was noted at a \( P_\mathrm{out} \) of 10 cm H\(_2\)O. At 16 weeks after tamoxifen treatment, only a small increase in sinus diameter was observed in vessels from Rasa1β/β Ubert2cre mice at the same pressure (Figure 5C). Valve leaflet measurements were made from the base of the valve sinus to the buttress of the valve leaflet at its point of attachment to the vessel wall (Figure 5A). In contrast with vessel wall and sinus diameter measurements, a significant reduction in valve leaflet length was observed at 9 weeks after tamoxifen treatment in vessels from Rasa1fl/fl Ubert2cre mice compared with Rasa1fl/fl controls and this reduction was also evident at 16 weeks (Figure 5D). These findings indicate that reduced valve leaflet length is the dominant factor responsible for impaired valve function in the absence of RASA1.

To gain further insight into the basis of LV valve dysfunction in the absence of RASA1, we conducted confocal microscopic imaging studies of popliteal LVs from Rasa1β/β and Rasa1β/β Ubert2cre mice. To identify valves, vessels were costained with antibodies against PROX1 and integrin α-9, both of which are more strongly
In parallel with reduced cellularity, popliteal LV valve leaflets in tamoxifen-treated $\text{Rasa1}^{fl/fl}$ $\text{Ubert2}^{cre}$ and $\text{Rasa1}^{fl/fl}$ $\text{Prox1}^{ert2cre}$ mice were shown to contain reduced amounts of ECM proteins, such as collagen IV and laminin $\alpha_5$, compared with littermate $\text{Rasa1}^{fl/fl}$ controls (Supplemental Figure 2).

Adult mice that express an R780Q knockin mutant form of RASA1 develop chylothorax. RASA1 is a prototypical negative regulator of Ras signaling that accelerates Ras hydrolysis of GTP (7). In addition, RASA1 has also been shown to act as an adapter protein that participates in cellular signal transduction in a manner unrelated to its ability to control Ras activation (7).
were administered tamoxifen, resulting in either expression of \( \text{Rasa1}^\text{R780Q} \) alone or complete loss of expression of \( \text{Rasa1} \) in the respective \( \text{Ubert2cre} \) strains. Starting at several weeks after tamoxifen administration, \( \text{Rasa1}^\text{fl/R780Q} \text{Ubert2cre} \) and \( \text{Rasa1}^\text{fl/fl} \text{Ubert2cre} \) mice both developed and succumbed to chylothorax (Figure 6B). Chylothorax was confirmed by flow cytometric analysis of pleural exudates that revealed a high content of T and B lymphocytes (Figure 6C). The time to development of chylothorax was similar between the 2 strains and perhaps even accelerated in \( \text{Rasa1}^\text{fl/R780Q} \text{Ubert2cre} \) mice. Potentially, accelerated chylothorax in \( \text{Rasa1}^\text{fl/R780Q} \text{Ubert2cre} \) mice could be explained on the grounds that the Cre recombinase needed only to cut and splice 1 rather than 2 \( \text{Rasa1}^\text{fl} \) alleles in order to create loss of functional RASA1 within LECs (Figure 6B). Staining of the chest wall of moribund \( \text{Rasa1}^\text{fl/R780Q} \text{Ubert2cre} \) mice with anti–LYVE-1 antibody revealed an abundance of LVs facing the pleural cavity, comparable to those observed in moribund \( \text{Rasa1}^\text{fl/fl} \text{Ubert2cre} \) mice.

Figure 8. Blocked LV valve development in induced RASA1-deficient and induced Rasa1R780Q embryos. Embryos of the indicated genotypes were administered tamoxifen at E15.5. Mesenteries were harvested at E19.5 and stained with antibodies against PROX1, CD31, and integrin α-9 to visualize LV valves. (A) Shown are merged confocal microscopic images of CD31 and PROX1 staining. Note lack of increased PROX1 staining at points of vessel bifurcation in \( \text{Rasa1}^\text{fl/fl} \text{Ubert2cre} \) and \( \text{Rasa1}^\text{fl/fl} \text{Ubert2cre} \) LVs. (B) Higher power images of PROX1 and integrin α-9 staining and merged images from lymphatics of \( \text{Rasa1}^\text{fl/fl} \) and \( \text{Rasa1}^\text{fl/R780Q} \text{Ubert2cre} \) mice. Note absence of integrin α-9 staining in the \( \text{Rasa1}^\text{fl/fl} \text{Ubert2cre} \) lymphatics. (C) Quantitation of LV valves in mesenteries of mice of the indicated genotypes (\( n = 3 \) each genotype). **** \( P < 0.0001 \), Student’s 2-sample t test.

ically, therefore, the lymphatic valve dysfunction that develops in induced RASA1-deficient mice could be consequent to loss of either function of RASA1. To address this, we used a \( \text{Rasa1}^\text{E7260Q} \) knockin mouse strain that we reported recently (12). Arginine 780 of RASA1 is the “arginine finger” of the GAP domain that is essential in order for RASA1 to promote Ras hydrolysis of GTP (26). Mutation of this residue to a glutamine abrogates catalytic activity, but leaves all other putative functions of RASA1 intact (27). Homozygous \( \text{Rasa1}^E7260Q \) mice die at E10.5 as a consequence of abnormal BV development (12). Thus, we generated heterozygous \( \text{Rasa1}^E7260Q \text{Ubert2cre} \) mice (Figure 6A). In the absence of tamoxifen, these mice develop normally and remain healthy throughout life, consistent with the fact that heterozygous \( \text{Rasa1}^{+/–} \) and \( \text{Rasa1}^E7260Q \) mice are also normal (10, 12). Mice were then crossed with \( \text{Rasa1}^{+/–} \text{Ubert2cre} \) mice to generate littermate \( \text{Rasa1}^E7260Q \text{Ubert2cre} \) and \( \text{Rasa1}^E7260Q \text{Ubert2cre} \) mice and corresponding \( \text{Rasa1}^{+/–} \text{Ubert2cre} \) and \( \text{Rasa1}^{+/–} \text{Ubert2cre} \) controls. At 3 to 4 weeks of age, mice were administered tamoxifen, resulting in either expression of \( \text{Rasa1}^E7260Q \) alone or complete loss of expression of \( \text{Rasa1} \) in the respective \( \text{Ubert2cre} \) strains. Starting at several weeks after tamoxifen administration, \( \text{Rasa1}^{+/–} \text{Ubert2cre} \) and \( \text{Rasa1}^{+/–} \text{Ubert2cre} \) mice both developed and succumbed to chylothorax (Figure 6B). Chylothorax was confirmed by flow cytometric analysis of pleural exudates that revealed a high content of T and B lymphocytes (Figure 6C). The time to development of chylothorax was similar between the 2 strains and perhaps even accelerated in \( \text{Rasa1}^{+/–} \text{Ubert2cre} \) mice. Potentially, accelerated chylothorax in \( \text{Rasa1}^{+/–} \text{Ubert2cre} \) mice could be explained on the grounds that the Cre recombinase needed only to cut and splice 1 rather than 2 \( \text{Rasa1}^{+/–} \) alleles in order to create loss of functional RASA1 within LECs (Figure 6B). Staining of the chest wall of moribund tamoxifen-treated \( \text{Rasa1}^{+/–} \text{Ubert2cre} \) mice with anti–LYVE-1 antibody revealed an abundance of LVs facing the pleural cavity, comparable to those observed in moribund \( \text{Rasa1}^{+/–} \text{Ubert2cre} \) mice.
that we reported previously (Figure 6D) (11). LVs are sparse in this region of control mice. In conclusion, findings indicate that the chylothorax and LV hyperplasia that develop in RASA1-deficient mice are primarily a consequence of loss of a Ras-regulating function of RASA1 in LECs.

LV valve dysfunction in induced Rasa1R780Q knockin mice. We next examined the pumping function of popliteal collecting LVs from Rasa1R780Q Ubert2cre−/− mice at different times after tamoxifen treatment. Vessels from mice treated with tamoxifen 5 and 12 weeks previously behaved similarly to vessels from tamoxifen-treated Rasa1fl/fl Ubert2cre−/− controls and had normal pump limits (Figure 6E). This is in contrast to vessels from Rasa1fl/fl Ubert2cre−/− mice that were unable to pump fluid against adverse pressure gradients when tested at 9 weeks after tamoxifen treatment (Figure 1B). However, when examined at 17 to 24 weeks after tamoxifen treatment, vessels from Rasa1fl/fl Ubert2cre−/− mice were also unable to pump against adverse pressure (Figure 6E). Thus, development of impaired pump function appears to be delayed in Rasa1fl/fl Ubert2cre−/− mice compared with Rasa1fl/fl Ubert2cre−/− mice.

To examine this further, we performed valve-closure and low-pressure back-leak tests on vessels from Rasa1fl/fl Ubert2cre−/− mice. At 12 weeks after tamoxifen treatment, 2 out of 4 tested valves behaved normally and closed in response to low adverse pressures across all tested initial vessel diameters. The remaining 2 valves showed a partial closure defect evidenced by a requirement for relatively high adverse pressures for closure at low vessel diameters (Figure 6F). However, across all vessel diameters, valves always closed within the range of adverse pressures that were examined. This is in contrast to valves from Rasa1fl/fl Ubert2cre−/− mice examined at 9 weeks after tamoxifen treatment, where all

**Figure 9. Apoptosis of PROX1+ LECs in developing LV valves of induced RASA1-deficient embryos.**

(A) Rasa1fl/fl and Rasa1fl/fl Ubert2cre−/− embryos were administered tamoxifen at E15.5. Mesenteries were harvested at E18.5 and stained with antibodies against PROX1, activated caspase 3, and PH3. Shown are confocal microscopic images of individual antibody stains of representative mesenteric LV valve regions from the 2 types of mice. Note that the majority of PROX1+ LECs in mesenteric LVs of Rasa1fl/fl Ubert2cre−/− embryos are stained with the anti-activated caspase 3 antibody and are in the process of apoptosis. (B) Percentage of apoptotic PROX1+ and PROX1− LECs in mesenteric LV valve regions of Rasa1fl/fl and Rasa1fl/fl Ubert2cre−/− embryos at E17.5 and E18.5 (n = 3 to 9 analyzed valve regions for each genotype and time point of analysis). ****P < 0.0001, Student’s 2-sample t test. See also Supplemental Figure 7, which shows LEC staining patterns at E17.5 and E19.5.
tested valves failed to close at any tested adverse pressure across all diameters (Figure 2, B and C). A valve-closure defect was more pronounced in Rasa1fl/fJR780Q Ubert2cre mice at 17 to 24 weeks after tamoxifen treatment. Thus, 4 out of 6 tested valves failed to close at any tested adverse pressure and diameter, 1 required abnormally high pressures to close at low vessel diameters and did not close at high vessel diameters, and 1 behaved normally (Figure 6F). All valves from control Rasa1fl/flR780Q mice treated with tamoxifen 16 to 25 weeks beforehand behaved normally in valve closure assays (Figure 6F).

Consistent with data from valve-closure tests, in low-pressure back-leak tests, valves from Rasa1fl/fJR780Q Ubert2cre mice treated with tamoxifen 5 and 12 weeks before were able to resist transfer of pressure to the upstream lumen (Figure 6G and Supplemental Video 8). In contrast, all 5 valves from Rasa1fl/fJR780Q Ubert2cre mice treated with tamoxifen 17 to 24 weeks previously that failed to close in valve-closure tests were unable to resist back-flow in low-pressure back-leak tests (Figure 6G). Analysis of video recordings of these tests confirmed that leakage was consequent to defective valve closing (Supplemental Video 9).

Reduced LV valve leaflet length largely accounts for valve dysfunction in induced RASA1-deficient mice. Popliteal LV valve leaflets from tamoxifen-treated Rasa1fl/flUbert2cre mice showed reduced length and cellularity compared with popliteal LV valve leaflets from tamoxifen-treated Rasa1fl/fl littermate control mice (Figure 5, D–F). To confirm the contribution of reduced leaflet length to valve dysfunction, we plotted mean leaflet length (average of the 2 leaflets) versus Psn pressure at a Pout of 10 cm H2O (Figure 7A). This analysis revealed a threshold mean leaflet length of popliteal LV valves of approximately 90–100 μm, below which valves were unable to resist back-flow. All valves from Rasa1fl/flUbert2cre mice treated with tamoxifen 9 and 16 weeks beforehand were at or below this threshold length and all were unable to resist back-flow. In contrast, all valves from control Rasa1fl/fl mice were at or above this threshold and all resisted back-flow. Valves from Rasa1fl/flUbert2cre mice treated with tamoxifen 1 week beforehand showed variability in ability to resist back-flow that largely correlated inversely with leaflet length, again centered at the 90-100 μm threshold (Figure 7A). An exception was 1 valve with a mean leaflet length of approximately 120 μm that showed moderate leakage (Figure 7A). Therefore, whereas in the majority of cases, valve leakage can be explained by reduced leaflet length, at an early time point after tamoxifen treatment, an additional mechanism may contribute to valve dysfunction.

To obtain information on the number of PROX1+ cells per valve leaflet that are required to maintain leaflet length above a functional threshold in popliteal LVs, we plotted PROX1+ cell numbers per leaflet against leaflet length (half of the leaflet outer perimeter) for different popliteal LV valves from Rasa1fl/fl and Rasa1fl/flUbert2cre mice (treated with tamoxifen 5 weeks earlier) as determined in confocal imaging experiments (Figure 5F). From a...
from Rasa1fl/fl Ubert2cre mice treated with tamoxifen 1 or 10 weeks beforehand, expression of both transcription factors and PROX1 in valve leaflet LECs was not diminished following loss of RASA1 (Supplemental Figure 3). Theoretically, reduced numbers of LECs in LV valves could be explained by reduced proliferation of leaflet LECs or loss of LECs from leaflets. By immunostaining using an antibody against phosphohistone H3 (PH3), we were unable to detect any proliferating LECs in valve leaflets or the vessel wall of popliteal LV valves of either Rasa1fl/fl or Rasa1fl/fl Ubert2cre mice following administration of tamoxifen (Supplemental Figure 3). Therefore, while we cannot formally exclude a contribution of reduced proliferation of leaflet LECs or vessel wall LECs to LV valve atrophy in induced RASA1-deficient mice, turnover of leaflet LECs even in adult RASA1-sufficient mice appears to be negligible. Loss of LECs from valve leaflets could be caused by impaired LEC intercellular adhesion or adhesion to the leaflet ECM core.

No difference in the expression level or distribution of the VE-cadherin and ZO1 cell-adhesion junction proteins, which are involved in linear regression analysis of this plot, it is estimated that functional popliteal LV valve leaflets (at or greater than 90–100 μm length) contain at least approximately 11–14 PROX1+ cells (Figure 7B).

A threshold functional valve length of 90–100 μm was also apparent in analyses of popliteal LV valves from tamoxifen-treated Rasa1fl/fl Prox1ert2cre mice (Figure 7C) and Rasa1fl/fl and Rasa1fl/fl Prox1ert2cre Ubrm2cre mice (Figure 7E). Furthermore, analyses of mesenteric LV valves from tamoxifen-treated Rasa1fl/fl and Rasa1fl/fl Prox1ert2cre mice revealed a similar threshold functional valve leaflet length (Figure 7D). One popliteal LV valve from a Rasa1fl/fl Prox1ert2cre mouse and 1 popliteal LV valve from a Rasa1fl/fl Prox1ert2cre Ubrm2cre mouse had mean leaflet lengths above the threshold, but still leaked (Figure 7, C and E). Thus, for these valves, leakage must also be explained by an alternative mechanism.

Expression of the transcription factors GATA-2 and FOXC2 in LV valve leaflet LECs has been shown to be required for maintenance of valve structure and function in adults (20, 23). However, as shown by immunostaining of sections of popliteal LV valves from Rasa1fl/fl Ubrm2cre mice treated with tamoxifen 1 or 10 weeks beforehand, expression of both transcription factors and PROX1 in valve leaflet LECs was not diminished following loss of RASA1 (Supplemental Figure 3). Theoretically, reduced numbers of LECs in LV valves could be explained by reduced proliferation of leaflet LECs or loss of LECs from leaflets. By immunostaining using an antibody against phosphohistone H3 (PH3), we were unable to detect any proliferating LECs in valve leaflets or the vessel wall of popliteal LV valves of either Rasa1fl/fl or Rasa1fl/fl Ubrm2cre mice following administration of tamoxifen (Supplemental Figure 3). Therefore, while we cannot formally exclude a contribution of reduced proliferation of leaflet LECs or vessel wall LECs to LV valve atrophy in induced RASA1-deficient mice, turnover of leaflet LECs even in adult RASA1-sufficient mice appears to be negligible. Loss of LECs from valve leaflets could be caused by impaired LEC intercellular adhesion or adhesion to the leaflet ECM core.

No difference in the expression level or distribution of the VE-cadherin and ZO1 cell-adhesion junction proteins, which are involved in...
in LEC intercellular adhesion, was observed for leaflet LECs between tamoxifen-treated Rasa1fl/ and Rasa1fl/Ubcrt2cre mice (Supplemental Figure 4 and data not shown) (28, 29). Furthermore, we did not observe any difference in the ability of cultured LECs from tamoxifen-treated Rasa1fl/ and Rasa1fl/Ubcrt2cre mice to adhere to the ECM proteins laminin, collagen, or fibronectin in vitro adhesion assays (Supplemental Figure 5). Therefore, any changes in the intrinsic ability of RASA1-deficient LECs to engage in intercellular or ECM adhesion are unlikely to account for LEC loss from leaflets.

A still further possibility that might explain LEC loss is apoptotic cell death following RASA1 loss. Given that 5 weeks after tamoxifen treatment, the average number of LECs in popliteal LV leaflets of Rasa1fl/Ubcrt2cre mice is reduced by less than 6 cells compared with Rasa1fl/mice, detection of LEC death in situ would be challenging (Figure 5F). In addition, it would be expected that apoptotic LECs would soon detach from leaflets as they are subjected to the shear forces of lymphatic flow. Nonetheless, through immunostaining of a large number of popliteal LV valves from tamoxifen-treated Rasa1fl/Ubcrt2cre mice, a single leaflet LEC was detected that expressed an activated form of the caspase 3 enzyme in the cytoplasm that characterizes cells in the early process of apoptosis (Supplemental Figure 6).

RASA1 catalytic activity is required for the development of LV valves. Since RASA1 is necessary for the maintenance of LV valves in adults, we also asked whether it is required for formation of LV valves during embryonic development. LV valvulogenesis in mice is initiated at E16.5 and is characterized by increased expression of PROX1 in valve-forming LECs, commonly at sites of vessel bifurcation (13, 16). Therefore, we administered tamoxifen to pregnant Rasa1fl dams carrying Rasa1fl/ and Rasa1fl/Ubcrt2cre embryos to disrupt Rasa1 expression in the latter in advance of normal valvulogenesis. Strikingly, administration of tamoxifen to embryos at any time from E12.5 to E14.5 resulted in complete absence of the LV system in E19.5 embryos as a result of LEC apoptosis that occurred between E17 and E18 (data not shown). Therefore, in subsequent experiments, we administered tamoxifen at E15.5, 1 day in advance of normal valvulogenesis. Administration of tamoxifen to Rasa1fl/Ubcrt2cre embryos at E15.5 did not result in LEC apoptosis, as assessed at E19.5. However, mesenteric LVs from these mice completely lacked valves, as indicated by the absence of LECs with increased PROX1 expression at earlier time points after tamoxifen treatment (Figure 9). At E19.5, PROX1hi LECs were absent from mesenteric LVs of Rasa1fl/Ubcrt2cre mice, and few apoptotic LECs were observed (Supplemental Figure 7). Very few apoptotic LECs were observed in mesenteric LVs of Rasa1fl/mice at any of the examined time points (Figure 9 and Supplemental Figure 7).

Within LV valve-forming regions, no significant difference in the number of PH3+ cells was noted between Rasa1fl/mice treated with tamoxifen at E15.5 or control mice at this time point (Figure 10A) (28, 29). Within LVs that were not subjected to tamoxifen treatment (Figure 9 and Supplemental Figure 7). Therefore, absence of LV valves at E19.5 in Rasa1fl/Ubcrt2cre mice treated with tamoxifen at E15.5 can be accounted for by death of PROX1hi LECs that occurs at E17.5 and E18.5. Coincident with apoptotic death of valve leaflet LECs at E17.5, the distribution of collagen IV in the ECM of developing valve leaflets was discontinuous in Rasa1fl/Ubcrt2cre mice compared with that observed in LV valves of Rasa1fl/mice at this time point (Figure 10A and Supplemental Figure 8). At E19.5, valve leaflet-associated luminal collagen IV projections observed in tamoxifen-treated Rasa1fl/mice could not be detected in tamoxifen-treated Rasa1fl/Ubcrt2cre mice in regions where valve formation would otherwise be expected (Figure 10B). However, lymphatic muscle coverage in these regions was comparable between the 2 types of mice (Figure 10B).

Loss of RASA1 within LECs results in impaired development of LV valves. To examine whether defects in LV valve development in tamoxifen-treated Rasa1fl/Ubcrt2cre embryos result from loss of RASA1 within LECs, we also examined LV valvulogenesis in Rasa1fl/Prox1rt2cre mice. In initial experiments, we discovered that administration of tamoxifen at E13.5 to Rasa1fl/Prox1rt2cre mice did not result in apoptotic death of all LECs, as observed in Rasa1fl/Ubcrt2cre embryos. Therefore, in all subsequent experiments, tamoxifen was administered at E13.5 to Rasa1fl/Prox1rt2cre mice, 3 days in advance of the onset of valvulogenesis. This strategy would permit a more robust assessment of the contribution of RASA1 to the earliest stages of LV valve development. Notably, at E17.5, PROX1 expression was increased in select LECs of Rasa1fl/Prox1rt2cre LVs (Figure 11A). This finding indicates that RASA1 is not required for early LV valvulogenesis. However, at E19.5, there were 75% fewer LV valves in Rasa1fl/Prox1rt2cre mice compared with Rasa1fl/mice (Figure 11, B and C). Therefore, loss of RASA1 specifically in LECs results in impaired development of mature LV valves. As in LVs from tamoxifen-treated Rasa1fl/Ubcrt2cre embryos, at E18.5, PROX1hi LECs in LVs from Rasa1fl/Prox1rt2cre mice but not Rasa1fl/mice embryos were frequently observed to stain with anti-activated
caspase 3 antibody, indicating apoptosis (Figure 11D). In addition, intraluminal projections of collagen IV were much less frequently observed in LVs of Rasa1fl/fl Prox1ert2cre embryos at E18.5 compared with controls, and for those valves that were identified at E18.5, the amount of collagen IV in valve leaflets was less compared with that in control valve leaflets (Figure 11E).

Discussion

Although CM-AVM was initially described as a BV disorder, a significant number of patients have now been reported with additional lymphatic abnormalities that include lymphatic leakage (chylothorax, chylos ascites, lymphedema) and lymphatic hyperplasia (2–6). Consistent with these reports, induced loss of RASA1 in adult mice also results in lymphatic leakage (chylothorax and chylos ascites) and hyperplasia (11). However, the origin of these lymphatic abnormalities in either species, particularly lymphatic leakage, has remained uncertain. Based on our earlier observation of retrograde flow of injected tracer dyes in NIRFLI studies, in the current study, we explored the possibility that RASA1 is required for the function of intraluminal valves that prevent lymph backflow in collecting LVs of normal individuals (13, 14). We show that in adult mice, induced loss of RASA1 results in dysfunctional valves that are unable to close properly and prevent back-leak in response to adverse intraluminal pressure. Consequently, RASA1-deficient collecting LVs are ineffective pumps despite the fact that the contractile activity of vessels is unaffected by RASA1 loss. The valve closure defect results from loss of RASA1 specifically within LECs and is explained by net loss of LECs from valve leaflets, resulting in leaflet shortening below a critical threshold length of 90–100 μm corresponding to 11–14 PROX1+ LECs per leaflet for afferent popliteal LVs. Adult mice that are induced to express R780Q RASA1 in the absence of WT RASA1 develop the same LV valve abnormalities, but with a delayed time of onset compared with induced RASA1-deficient mice. This finding indicates that RASA1 contributes to LV valve function both by acting as a negative regulator of Ras and through participation in other distinct signaling pathways. In addition to its requirement for normal valve function in adults, RASA1 expressed within LEC was shown to be necessary for LV valve development in late embryogenesis. However, in contrast to valve maintenance, no evidence was obtained of a role for RASA1 in valve development that was independent of its GAP activity.

The finding that RASA1 is required for valve function and development provides at least part of an explanation for the lymphatic leakage defects that are observed in CM-AVM patients and RASA1-deficient mice. Furthermore, it is possible that at least part of the LV hyperplasia observed in both species is secondary to the valve defect and represents a homeostatic lymphangiogenic response intended to absorb accumulating interstitial fluid or lymph in tissues or body cavities. In agreement with this possibility, valve defects can be detected as soon as 1 week after RASA1 loss (Figure 3). In contrast, as shown in NIRFLI studies, the earliest time that LV hyperplasia can be detected is 2 weeks after gene disruption (4, 11). Furthermore, in light of the present findings, it is possible that any apparent hyperplasia at 2 weeks in NIRFLI studies does not represent hyperplasia per se, but back flow of dye into preexisting LVs. To obtain additional evidence that valve defects precede the onset of hyperplasia, we stained diaphragms from the same mice that exhibited severe defects in mesenteric LV valve function (Figure 4) with antibodies against PROX1 and CD31. In these mice, LV hyperplasia was not apparent (Supplemental Figure 9).

LV hyperplasia in induced RASA1-deficient mice can be inhibited by continuous administration of blocking anti-VEGFR3 antibodies to mice (11). Our earlier interpretation of this finding is that RASA1 normally functions as a negative regulator of VEGFR3 signaling that prevents Ras activation in LV wall LECs triggered by its ligand VEGF-C, present at low concentrations in the extracellular space in unchallenged animals. In this interpretation, in the absence of RASA1, these low concentrations of VEGF-C would result in chronic activation of Ras in LECs and lymphangiogenesis. However, it is conceivable that dysregulated signaling through VEGFR3 on valve leaflet LECs in induced RASA1-deficient mice also contributes to the development of hyperplasia. Indeed, VEGFR3 is expressed at substantially higher levels on LECs in LV leaflets than on vessel wall LECs (30).

The notion that LV abnormalities in CM-AVM are primarily a result of valve dysfunction may also explain why LV abnormalities are restricted to a subset of patients. Presumably, in order for valve dysfunction or failed valve development to manifest, a critical number of valve leaflet LECs or valve-forming cells, respectively, would have to harbor inactivating second-hit mutations in the normal WT RASA1 allele. As a rare event, this is unlikely to be accomplished unless such mutations are acquired at an early point in vasculogenesis and specifically in cells that can give rise to valve-forming cells. Conversely, BV malformations could arise more frequently after second-hit mutation of RASA1 based on a less stringent requirement for multiple endothelial cells in a specific location to be affected. Whether impaired valve function and development is alone responsible for LV leakage in CM-AVM patients and induced RASA1-deficient mice remains to be determined. In this regard, it is of note that, although development of valve dysfunction is delayed in induced Rasa1R780Q mice compared with induced RASA1-deficient mice, the development of chylothorax occurs with similar kinetics and is perhaps even accelerated in the Rasa1R780Q mice.

RASA1 does not appear to be required for the initial increased expression of PROX1 in early LV valvulogenesis (Figure 11). Rather, RASA1 is required for the survival of PROX1+ LECs in developing valves (Figures 9 and 11). Apoptotic death of PROX1+ LECs during LV valvulogenesis thus explains the absence of mature LV valves in induced RASA1-deficient embryos. Based upon this finding, it is likely that the net loss of LECs in LV valve leaflets of adult induced RASA1-deficient mice also results from increased LEC apoptotic death. This would be difficult to detect in vivo given the small number of LECs in each valve leaflet (average of 13 in valves of afferent popliteal collecting LVs) and the fact that LEC loss from leaflets is a slow process (loss of 5 to 6 LECs per leaflet over 5 weeks). Potentially, LV wall LECs could show similar increased susceptibility to apoptosis following loss of RASA1. However, LV wall LECs would not be subject to the increased shear forces experienced by valve leaflet LECs that might be necessary for net LEC loss in vivo.

The mechanism by which RASA1 loss results in apoptosis of LECs in developing and, potentially, mature valves is uncertain. One possibility is LEC detachment from the ECM that would trigger
apoptotic death, a process referred to as anoikis (31). Detachment from the ECM could be caused by changes in the avidity of LEC adhesion receptors for ECM proteins. However, we were unable to detect any alteration in the ability of RASA1-deficient LECs to adhere to ECM proteins in vitro. Alternatively, detachment from the ECM could be caused by decreased synthesis or increased degradation of ECM proteins. This would be consistent with our finding of reduced amounts of collagen IV in developing valve leaflets and mature valves following loss of RASA1, although whether this is a cause of or consequence of leaflet LEC apoptosis is unknown. Another possibility is that RASA1 regulates LEC apoptosis directly. Interestingly, an anti-apoptotic function of RASA1, which is mediated by an N-terminal cleavage fragment of the molecule, has been reported previously (32, 33). However, it is unknown whether this anti-apoptotic function of RASA1 requires GAP activity.

Finally, the findings reported here raise the possibility that defective valve function is the underlying cause of LV abnormalities in several different syndromic conditions known as RASopathyes that are caused by inherited mutations in different genes that result in increased Ras signal transduction. These include Noonan syndrome (PTPN11, SOS1, KRAS, or RAF1 mutations), cardiofaciocutaneous syndrome (KRAS mutation), and Costello syndrome (HRAS mutation) (34, 35). LV abnormalities such as lymphedema, chyloous ascites, and chylothorax are common in these diseases (36). In support of this possibility, in recent imaging studies of Noonan syndrome and cardiofaciocutaneous syndrome patients, retrograde flow of lymph in collecting LVs was observed frequently (37).

**Methods**

For a full description of Methods, see Supplemental Methods.

**Mice.** Rasa1(−/−) mice with and without Ubr2(−/−) or Proxl(−/−) transgenes and Rasa1(ΔR780Q) mice have been described (11, 12, 38). The Proxl(−/−) transgenic line was provided by G. Oliver (Northwestern University Medical School, Chicago, Illinois, USA). Rasa1(ΔR780Q) Ubr2(−/−) mice were generated through crossbreeding. All mice were on a mixed 129S6/SvEv x C57BL/6 genetic background.

**Statistics.** Statistical analysis was performed using Student’s 2-sample t test. A P value of less than 0.05 was considered significant.

**Study approval.** All experiments performed with mice were in compliance with University of Michigan and University of Missouri guidelines and were approved by the respective university committees on the use and care of animals.

**Author contributions**

All authors contributed to the design of studies and conducted experiments. The manuscript was written by MJD and PDK.

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17. Polito A, et al. Vascular system abnormalities in mice lacking an N-terminal cleavage fragment of the molecule, has been reported previously (32, 33). However, it is unknown whether this anti-apoptotic function of RASA1 requires GAP activity.

Finally, the findings reported here raise the possibility that defective valve function is the underlying cause of LV abnormalities in several different syndromic conditions known as RASopathyes that are caused by inherited mutations in different genes that result in increased Ras signal transduction. These include Noonan syndrome (PTPN11, SOS1, KRAS, or RAF1 mutations), cardiofaciocutaneous syndrome (KRAS mutation), and Costello syndrome (HRAS mutation) (34, 35). LV abnormalities such as lymphedema, chyloous ascites, and chylothorax are common in these diseases (36). In support of this possibility, in recent imaging studies of Noonan syndrome and cardiofaciocutaneous syndrome patients, retrograde flow of lymph in collecting LVs was observed frequently (37).


