Title

Human venous valve disease caused by mutations in *FOXC2* and *GJC2*

Short caption

Venous valves ensure unidirectional blood flow in veins. Lyons et al. describe imaging of valves and show that patients with mutations in *FOXC2* and *GJC2* have reduced venous valve number and leaflet length. In mice, Foxc2-Calcineurin-Nfatc1, and *Gja4*, *Gjc2*, *Gja1* critically regulate valve-forming cell organisation. Foxc2, Calcineurin-Nfatc1 and blood flow regulate leaflet growth/maturation.

Authors

Oliver Lyons[1], Prakash Saha[1], Christopher Seet[1], Adam Kuchta[2], Andrew Arnold[2], Steven Grover[3], Victoria Rashbrook[1], Amélie Sabine[4], Gema Vizcay-Barrena[5], Ash Patel[1], Francesca Ludwinski[1], Soundrie Padayachee[2], Tsutomu Kume[6], Brenda R. Kwak[7], Glen Brice[8], Sahar Mansour[8], Pia Ostergaard[9], Peter Mortimer[9], Steve Jeffery[9], Nigel Brown[10], Taija Makinen[11], Tatiana V. Petrova[4], Bijan Modarai[1], Alberto Smith[1]

Affiliations

1. Academic Department of Vascular Surgery, Cardiovascular Division, BHF Centre of Research Excellence, King's College London, St Thomas' Hospital, United Kingdom
2. Department of Ultrasonic Angiology, Guy's & St Thomas' NHS Foundation Trust, United Kingdom
3. Division of Hemostasis and Thrombosis, Beth Israel Deaconess Medical Center and Harvard Medical School, USA
4. Department of Fundamental Oncology, Ludwig Institute for Cancer Research and Division of Experimental Pathology, Centre Hospitalier Universitaire Vaudois and University of Lausanne, Epalinges, Switzerland
5. Center for Ultrastructural Imaging, King's College London, United Kingdom
6. Feinberg Cardiovascular Research Institute, Northwestern University School of Medicine, USA
7. Department of Pathology and Immunology, University of Geneva, Switzerland
8. SW Thames Regional Genetics Service, St George's Hospital, United Kingdom
9. Cardiovascular and Cell Sciences Institute, St George's University of London, UK
10. Institute of Medical & Biomedical Education, St George's University of London, United Kingdom
11. Rudbeck Laboratory, Department of Immunology, Genetics and Pathology, Uppsala University, Sweden

Corresponding authors
Dr Oliver Lyons and Prof Alberto Smith
Academic Department of Vascular Surgery, KCL
1st Floor North Wing
St Thomas' Hospital
London, SE1 7EH
United Kingdom,
Email:oliver.lyons@kcl.ac.uk and alberto.smith@kcl.ac.uk

ORCID
Dr Lyons: orcid.org/0000-0002-7731-7343
Prof Smith: orcid.org/0000-0002-3042-5099

Summary (Approx 40 words)
Patients with mutations in *FOXC2* and *GJC2* have reduced venous valve number and leaflet length. Experiments in mice show that Foxc2-Calcineurin-Nfatc1, and *Gja4*, *Gjc2*, *Gja1* regulate valve-forming cell organisation. Foxc2, Calcineurin-Nfatc1, and blood flow regulate leaflet growth/maturation.

Acknowledgements
Funding was provided by an NIHR Academic Clinical Fellowship, MRC Clinical Research Fellowship (G1000327) and Centenary Award, and BHF Centre of Excellence grant to OL/AS. This work was supported by a Sinergia grant from the Swiss National Science Foundation (FN31ER30-160674 to TVP and CRSII3-141811 to TVP and BRK). We would like to thank Dr Brant Isakson for advice on TEM and Bushra Rais for assistance with immunohistochemistry. The authors declare no competing financial interests.

Author contributions
OL, PS, CS, AK, AA, SP, SJ, NB, TM, TP, BM, AS designed research studies; OL, CS, AK, AA, SG, AP, SP, VR, GVB conducted experiments, OL, PS, CS, AK, AA, FL, SP acquired & analysed data, GB, SM, PO, PM, SJ provided patients and all authors wrote and approved the manuscript.

Non-standard abbreviations

Cx37 Connexin37
Cx43 Connexin43
Cx47 Connexin47
FV Femoral vein
LV Lymphatic valve
VFC Valve-forming cell

Abstract (162 words)

Venous valves (VVs) prevent venous hypertension and ulceration. We report that *FOXC2* and *GJC2* mutations are associated with reduced VV number and length. In mice, VV initiation is marked by elongation and reorientation ("organisation") of Prox1^{hi} endothelial cells by postnatal day 0. The expression of the transcription factors *Foxc2* and *Nfatc1,* and the gap junction proteins, *Gjc2, Gja1* and *Gja4,* were temporo-spatially regulated during this process. Foxc2 and Nfatc1 were co-expressed at P0, and combined *Foxc2* deletion with calcineurin-Nfat inhibition disrupted early Prox1^{hi} endothelial organisation, suggesting co-operative Foxc2-Nfatc1 patterning of these events. Genetic deletion of *Gjc2, Gja4* or *Gja1* also disrupted early VV Prox1^{hi} endothelial organisation at P0, and this likely underlies the VV defects seen in *GJC2*-mutated patients. Knockout of *Gja4* or *Gjc2* resulted in reduced proliferation of Prox1^{hi} valve-forming cells. At later stages blood flow, Foxc2 and calcineurin-Nfat signalling are each required for growth of the valve leaflets, while Foxc2 is not required for VV maintenance.

Introduction

Venous valves (**VV**s) are widely distributed throughout veins and venules, and facilitate unidirectional blood flow back to the heart, which acts to reduce the peripheral venous blood pressure.(Bergan et al., 2006; Meissner et al., 2007; Phillips et al., 2004) VV failure is a central feature of the venous reflux that is seen in up to 40% of adults. Reflux leads to chronic venous hypertension (particularly in the lower limbs) which can cause pain, oedema, hyperpigmentation, skin damage, and chronic intractable ulceration.(Bergan et al., 2006; Meissner et al., 2007) Our understanding of the molecular mechanisms of VV development and subsequent maintenance is limited, and there are few therapeutic options to treat VV dysfunction.(Bazigou et al., 2011; Bergan et al., 2006; Caggiati, 2013; Maleti et al., 2015; Munger et al., 2016; Munger et al., 2013) Elucidation of these mechanisms and understanding of how their dysfunction may lead to VV failure could facilitate the development of novel therapies to treat this condition.

Clinical studies have suggested a link between venous reflux and some primary lymphoedemas, but the cause of this, (i.e. a direct VV defect or an indirect effect such as vein dilatation) has not been elucidated.(Mellor et al., 2007; Ostergaard et al., 2011; Rosbotham et al., 2000) We have previously shown how genes (*Itga9*, *Efnb2*, *Fn-EIIIA*) regulating lymphangiogenesis also control VV formation and maintenance.(Bazigou et al., 2011) A number of transcription factors (Prox1, Foxc2, Nfatc1) and gap junction proteins (connexin37, connexin43 and connexin47) have been implicated in the development of lymphatic (**LV**), cardiac or venous valves.(Chang et al., 2004; de la Pompa et al., 1998; Kanady et al., 2011; Molica et al., 2014; Munger et al., 2016; Munger et al., 2013; Norrmen et al., 2009; Petrova et al., 2004; Srinivasan.RS, 2011; Winnier et al., 1999) Mutations in the genes encoding FOXC2, (*FOXC2*) Connexin47 (**Cx47**, *GJC2*) and Connexin43 (**Cx43**, *GJA1*) cause primary lymphoedema in man.(Brice et al., 2013; Fang et al., 2000; Ferrell et al., 2010; Ostergaard et al., 2011) *Gjc2*$^{-/-}$ and *Gja4*$^{-/-}$ (**Cx37**) mice have VV defects during VV maturation, but the timing of onset of these abnormalities during VV formation and the developmental processes underlying absent VVs have not been studied.(Munger et al., 2016; Munger et al., 2013) Expression of Foxc2 and Nfatc1 is segregated to opposite leaflet surfaces during valve maturation, but their expression during VV initiation, and potential co-operative signalling (as is seen in lymphatic endothelia), has not been studied.(Munger et al., 2016; Norrmen et al., 2009) Similarly, during leaflet maturation Cx37 and Nfatc1 are not co-expressed in the same valve leaflet ECs, but expression patterns in VV initiation remain undetermined.(Munger et al., 2016)

Here, we quantify the VV defects in the limbs of patients with primary lymphoedema caused by mutations in *GJC2* or *FOXC2* and examine the earliest underlying mechanisms of VV failure caused by loss of these genes in mice. We identify reduced numbers of VVs, and shorter VV leaflets, in patients with lymphoedema caused by mutations in *FOXC2* or *GJC2*. In mice, we characterise the induction and organisation of valve-forming endothelial cells (**VFC**s) which occurs at an earlier time point than previously identified, and show that this occurs within highly spatially and temporally regulated domains of Prox1, Foxc2, Nfatc1, Cx37, Cx43 and Cx47 expression.(Bazigou

et al., 2011; Munger et al., 2016) Combined *Foxc2* deletion and inhibition of Nfat signaling (but not *Foxc2* deletion or Nfat inhibition alone) resulted in the failure of early VFC organisation. Loss of connexin-encoding genes *Gja1, Gjc2* or *Gja4* similarly resulted in a failure of VFC organisation, and for *Gjc2* and *Gja4* this resulted in reduced VFC proliferation. Both Nfat and Foxc2 signaling and blood flow were required for VV maturation to postnatal day 6 (P6). Unlike its role in LVs, we show that Foxc2 is not required in VV EC's for valve maintenance.(Sabine et al., 2015)

Results

FOXC2, *GJC2* and *GJA1* mutations in human VV disease

Understanding the relationship between primary lymphoedema and VV dysfunction has been hampered by our inability to noninvasively quantify the presence and morphology of VVs in patients. Whilst VVs have been seen by ultrasonography, they have not been quantified on a systemic basis.(Lane, 2007; Lurie and Kistner, 2012). We used conventional ultrasonography to visualise VVs in man (Fig.1A). Quantification was reproducible for the number of VV/vein in repeated scanning by different operators (N=28 veins, intraclass correlation coefficient =0.90, 95%CI 0.63-0.91, P<0.0005) and for leaflet length in repeated measurement by different operators (N=15 VV, intra-class correlation coefficient =0.94, 95%CI 0.83-0.98, P<0.0005). This allowed quantification of the number of VVs and VV leaflet length in the upper and lower limbs (Suppl. Tables 1,2). To limit scan duration, we scanned only the short saphenous, popliteal, basilic and brachial veins. Consistent with post-mortem studies, different numbers of VVs (Suppl. Tables 2,3) were detected in control veins (mean±SD, popliteal 1.1±0.7; short saphenous 3.4±1.5; brachial 1.8±1.2; basilic 2.4±0.9, P<0.0005, ANOVA) and as expected, with different leaflet lengths (popliteal 6.8±1.9mm; short saphenous 4.0±1.4mm; brachial 3.9±1.6mm; basilic 4.9±2.2mm; P<0.0005, ANOVA).(Czarniawska-Grzesinska, 2002; Iimura et al., 2003; Moore et al., 2011; Schweighofer et al., 2010) Subsequent comparisons were therefore analysed as a fold change relative to the respective vein in controls.

We found significantly reduced numbers of VVs in patients with mutations in both *FOXC2* (fold change±SD: 0.25±0.32, P<0.0005) and *GJC2* (0.38±0.46, P<0.0005, Fig. 1, Tables 1,2) compared with age and sex-matched controls. Those VVs that were present in the *FOXC2* and *GJC2* mutation patients had shorter leaflets (0.44±0.25, P<0.0005 and 0.61±0.28, P<0.0005 respectively). VV defects were present in veins of both upper and lower limbs in patients for both *FOXC2* and *GJC2* (data not shown). There also appeared to be fewer VVs in the single patient identified with lymphoedema caused by a mutation in *GJA1* (connexin43). Consistent with these findings, and in agreement with their expression pattern in murine VVs, FOXC2, and CX43 were localised on adult human VVs, suggesting that human VV phenotypes may result from tissue-autonomous effects of patient's mutations (Fig. 1D). CX43 predominantly located on the lumen-facing leaflet surface, whilst FOXC2 predominantly located on the sinus surface. We were unable to obtain typical punctate CX47 staining using commercially available antibodies in human VVs. It is possible that the Cx47 expressed in VV during development and early life is subsequently down regulated in the adult human VV, as has been found in mice for VEGFR3 (Bazigou et al.)

Imaging early VV development in mice

To investigate possible underlying mechanisms for these human phenotypes, we next analysed VV development in mice and focused on the proximal femoral vein (**FV**)

because of the critical role of proximal deep VV in human disease, and because a VV most consistently develops at this position in the mouse, allowing reliable identification of the earliest stages in its development.(Bazigou et al., 2011; Eberhardt and Raffetto, 2014; Meissner et al., 2007) We had previously analysed *opened* veins (by electron and confocal microscopy), which damages VV structures and precluded analysis of the anterior vein wall and complete staging of VV development.(Bazigou et al., 2011) We therefore used whole-mount confocal microscopy of *unopened* veins to visualise the proximal FV prior to and during VV formation.

Analysis from E17 to P6 revealed that initiation of VV formation occurs earlier than previously identified by imaging of opened veins or frozen sections (Fig. 2A-C).(Bazigou et al., 2011; Munger et al., 2016) At E17, Prox1 was initially widely expressed throughout the FV (data not shown), with increased expression in a heterogeneous subset of endothelial cells, termed Prox1^{hi} cells (as in LV).(Sabine et al., 2012; Sweet et al., 2015) These cells were distributed across the vein in the region of prospective VV formation (Fig. 2C,D Stage 0 of development). At P0 Prox1^{hi} nuclei had undergone elongation and reorientation and had organised to form a ring of VFCs around the vein (Stage 1). Organisation of VFCs is more prominent on the anterior wall of the FV, prior to full circularisation (Fig.2F,G). This ring structure is the first time point when the contiguous ring of spindle-shaped cells at the free edge of the valve leaflets (that is present throughout development and in adults) becomes identifiable. From this ring, a roughly circular leaflet develops toward the center of the FV, forming a marked constriction (Stage 2). Part of the free-edge ring extends to the vessel wall to form the first commissure (Stage 3). The free edge opposite this first commissure is not in contact with the vessel wall (see also Suppl. Fig 1A, BALB/C). This is followed by formation of a second commissure (Stage 4). At earlier stages smooth muscle cells uniformly coat the FV but subsequently become sparser in the region of the VV sinus (Fig. 2E at P6).

Foxc2 & Nfatc1 co-operate to pattern initial organisation

The mechanism(s) by which mutations in *FOXC2* and *GJC2* lead to defective VV are unknown.(Mellor et al., 2007; Petrova et al., 2004) We first analysed the regulation of the initial ring of VFCs (Stage 1) that forms by P0. *Foxc2*$^{-/-}$ mice do not survive to birth so we investigated the effect of conditional deletion of *Foxc2* using tamoxifen-inducible Cre recombinase under the control of the *Prox1* promoter (*Prox1;CreER*^{T2}) in combination with floxed target alleles.(Bazigou et al., 2011) Induction of Cre activity at E15 induced deletion in VV cells. However, neither heterozygous (*Foxc2*$^{lx/+}$) or homozygous (*Foxc2*$^{lx/lx}$) deletion produced a significant phenotype at P0 (Fig. 3B). We therefore analysed expression of the transcription factor Nfatc1, which is co-expressed with Foxc2 and is known to signal cooperatively during lymphatic vessel maturation.(Norrmen et al., 2009) At P0, Nfatc1 was co-localised with Foxc2 in Prox1^{hi} VFCs and predominantly downstream of the forming valve (Fig. 3A). Blocking Nfat signaling by homozygous deletion of the regulatory subunit of calcineurin (CnB1, encoded by *Ppp3r1*) in venous endothelium from E15, or administration of Cyclosporin A (CsA, a well characterised pharmacological inhibitor of Calcineurin-Nfat signalling) from E17 resulted in the expected cytoplasmic localization of Nfatc1 (Suppl. Fig. 2A-

C), but had no effect on VFC organisation at P0 (Fig. 3C,D).(Chang et al., 2004; Norrmen et al., 2009; Sabine et al., 2012) To simultaneously inhibit signaling through both transcription factors, we treated *Prox1CreER^{T2};Foxc2$^{lx/lx}$* mice with CsA. Combined *Foxc2$^{lx/lx}$* deletion and Nfat inhibition resulted in significantly abnormal VFC organisation (Fig. 3E). Thus, *unlike* lymphatic valves, only combined inactivation of Foxc2 and calcineurin/Nfat signaling was able to disrupt normal VV organisation.(Norrmen et al., 2009)

Connexins pattern VV organisation at P0

We next analysed the expression of Cx37, Cx43, and Cx47, at the earliest stages of VV development in the FV (Fig. 4A,C,E) At E18, Cx37 was immunolocalised to lymphatics (data not shown) but was *not* detectable in the region of the FV where a VV subsequently always develops (Suppl. Fig. 3A). At P0, Cx37 was localised to VFCs, particularly in the upper and lower region of the vessel (Fig. 4A). In *Gja4* (Cx37) homozygous constitutive knockout mice, initiation of VV formation at the usual location, and the typical boundary between higher distal Prox1 immunostaining and higher proximal Foxc2 immunostaining was preserved. VFCs were identified distributed across the full width of the vessel but appeared highly disorganized, failing to reach Stage 1, whilst all WT and heterozygous knockout littermate VVs reached Stage 1 as expected (Fig. 4B). We next analysed the junctions between VFCs by TEM at P0 and, despite the rearrangements that these cells are undergoing, confirmed the appearance of gap junctions (inset in Fig. 4G, Suppl. Fig. 3J,K). Whilst gap junction-mediated intercellular communication has been implicated in the regulation of Nfatc1 signaling in LV development *in vitro*, Nfatc1 immunolocalisation remained nuclear in *Gja4$^{-/-}$* mice, demonstrating that Cx37 is not critically required upstream of Nfat nuclear translocation in VV, albeit Nfatc1^{hi} nuclei were disorganized (as seen with Prox1 immunolocalisation. Suppl. Fig. 3D).(Kumai et al., 2000; Sabine et al.)

In contrast to Cx37, expression of Cx43 was not detectable on the vast majority of VFCs, but was instead localised to endothelial cells upstream of these cells, with the strongest Cx43 immunostaining in the superior half of the upstream vessel (Fig. 4C). It is possible that junctions consisting of Cx43 allow communication between this upstream region and VFCs in some areas. The source of Cx43 at junctions between these cells (either VFC or upstream endothelia) remains unclear. Homozygous conditional deletion of *Gja1* (Cx43) resulted in failure of organisation of the VFCs downstream of this region, and VVs failed to reach Stage 1, whilst wild-type littermates developed normally (Fig. 4D).

Cx47 expression was studied using a *Gjc2^{GFP}* (knock in) reporter, with signal amplification using antibodies raised against GFP. Whilst no GFP signal was detected in the FVs of wild-type mice, expression was detected in cells dispersed throughout the FV in *Gjc2$^{GFP/+}$* mice (Fig. 4E, Suppl. Fig. 3G,H) and *Gjc2$^{GFP/GFP}$* mice (Suppl. Fig. 3I). Compared with wild type littermates, Prox1^{hi} VFCs showed a pattern of disorganisation similar to loss of *Gja4* and *Gja1* at P0, but, we could find very few VFCs expressing detectable Cx47 (GFP, Fig. 4F), suggesting a non VFC-autonomous role of *Gjc2* in modulating VFC organisation.

In order to further quantify the phenotypes resulting from loss of Cx37, Cx43 or Cx47, we first used *Prox1CreER^{T2}* crossed with the *Rosa26mTmG* reporter, in which membrane-bound GFP is expressed in Cre-recombined cells, enabling us to show that nuclear morphology (orientation relative to the vessel, and length:width ratio) can be used as a surrogate marker for cellular morphology in P0 VFCs (Suppl. Fig. 4A,B).(Muzumdar et al., 2007) We quantified Prox1^{hi} nuclear elongation and reorientation as components of VV organisation (Fig. 5A-A''). Elongation did not correlate with reorientation (Suppl. Fig. 4C), and we therefore analysed these attributes separately. Elongated Prox1^{hi} nuclei were significantly more reoriented in the central third of the vessel than the upper and lower thirds (Fig. 5A'', Suppl. Fig. 4D). We analysed the proportions of Prox1^{hi} nuclei in each region of the vein, where loss of Cx37 (but not Cx43 or Cx47) resulted in a reduced proportion of Prox1^{hi} nuclei in the center of the vessel (Fig. 5B). To quantify organisation, we analysed elongation and reorientation of nuclei within the central tertile, as previously described.(Tatin et al., 2013) Loss of Cx37 or Cx43 resulted in reduced nuclear elongation, whilst loss of Cx37 or Cx43 or Cx47 resulted in reduced reorientation of nuclei (Fig. 5; data for all genotypes and vessel regions analysed are provided in Suppl. Fig. 4E). Together, these data show that Cx37, Cx43 and Cx47 are each critical for the cellular reorganization that occurs in order to reach stage 1 of VV development.

To determine whether connexin loss resulted in failed proliferation of VFCs, we analysed the proportion of Prox1^{hi} VFCs co-labelled with Ki67. This revealed reductions in the proportion of proliferating VFCs in both *Gja4$^{-/-}$* and *Gjc2$^{GFP/GFP}$* mice ($P<0.0005$ and $P<0.005$, respectively, Fig. 5E,F). We could find no evidence of apoptotic VFCs in either of these knockout lines (data not shown).

Maturation of leaflets and commissures

We next analysed the maturation of VV to P6, and asked whether Foxc2 or Calcineurin-Nfat signaling are required for development of VV leaflets and commissures. In contrast to their co-operative roles in patterning the initial VV ring, calcineurin inhibition with CsA (Fig. 6A), or loss of CnB1 (*Ppp3r1$^{lx/lx}$* deletion) at P0 (Fig. 6B,C), or deletion of *Foxc2* alone (Fig. 6D), were each sufficient to cause significant defects in leaflet/commissure development through to stages 3-4. There was however no loss of the ring of rotated Prox1^{hi} free-edge cells, which remained intact even at P6 (Fig. 6A,B), demonstrating that neither Nfatc1 signaling or Foxc2 are required for maintenance of this initial valve structure and the free-edge phenotype of these cells.

Cx37 remained expressed in VV leaflets at P6 (Fig. 6E). Unlike the phenotype seen with loss of *Ppp3r1*, *Foxc2*, and to some extent *Itga9* or *Efnb2,* (Suppl. Fig. 5) analysis of *Gja4$^{-/-}$* VVs at P6 revealed complete absence of both leaflet structure and Prox1^{hi} or Foxc2^{hi} cells (Fig. 6F), demonstrating that the early development of VV seen at P0 in *Gja4$^{-/-}$* (Cx37) mice is subsequently lost, and Cx37 is required to maintain the free-edge cell phenotype. This was associated with failure to form the gap in SMA-expressing cells normally seen around the valve (6F). As expected, VV in *Gja4$^{+/-}$* mice developed normally (Suppl. Fig. 5G). These results are consistent with previously reported

findings in LVs and with the absence of VVs at P4 in *Gja4*$^{-/-}$ mice.(Munger et al., 2016; Munger et al., 2013)

At P2, Cx43 was barely detectable in VV leaflets (Suppl. Fig. 5H), but at P6 was clearly localised around Nfatc1-expressing ECs (Fig. 6E). Conditional homozygous *Gja1* deletion from P0 did not produce a significant phenotype at P6, suggesting that the critical requirement for Cx43 in VV development is restricted to events prior to or around P0 (Suppl. Fig. 5I).

GFP-expressing cells were almost entirely undetectable in *Gjc2*$^{GFP/+}$ VV at P2 (Suppl. Fig. 5K), but by P6, Cx47-expressing cells were clearly detected within the valve (Fig. 6J). In contrast, *Gjc2*$^{GFP/GFP}$ mice (lacking Gjc2 expression) had no valve cells by P2, indicating loss of the Prox1^{hi} phenotype seen at P0. These results suggest that the critical requirement for Cx47 is restricted to events around P0 (Fig. 6I, Suppl. Fig. 5L), and are in agreement with previous findings of absent VV in femoral and other veins analysed at later postnatal stages in *Gjc2*$^{-/-}$ mice.(Munger et al., 2016) Notably, Cx47 is not required for the development of valves in all veins (for example the superficial caudal epigastric or proximal subclavian veins), consistent with our finding of approximately 40% VV remaining in peripheral veins of patients with mutated *GJC2*.(Munger et al. 2016)

A role for blood flow in VV development

Because several genes required for VV development, including *Foxc2* and *Cx37*, are upregulated by fluid shear stress, we hypothesized that blood flow may be required for normal VV leaflet growth.(Sabine et al., 2012; Sweet et al., 2015) To alter blood flow across the developing VV we ligated and divided the FV at P0, and analysed the VV at P6. No thrombosis was seen and operated pups gained weight normally (P>0.05 vs unoperated littermates). We initially ligated the FV at P0 (Fig. 7A), which did not result in visible diversion of blood flow (likely due to the small collaterals seen at the site of the ligation; not shown) and valve development was unaffected. We then ligated the FV twice and divided the FV (Fig. 7B), resulting in re-routing of blood via collaterals, with flow past the main valve region seen to re-enter via these collaterals (Fig. 7B). VV exposed to these altered flow conditions showed reduced progression to the later stages of leaflet development compared with VV exposed to unaltered flow conditions on the unoperated contralateral side (Fig. 7B) and were smaller (Fig. 7C).

Regulation of VV maintenance

Because heterozygous *FOXC2*-mutated patients showed severe VV defects, and *Foxc2* is required for LV maintenance, we next asked whether *Foxc2* is also needed for VV maintenance as well as development.(Sabine et al., 2015) Induction of deletion in *Foxc2*$^{lx/lx}$ mice at 4weeks resulted in absent Foxc2 immunosignal 4days later (data not shown), but interestingly VV length at 6weeks (*Foxc2*$^{lx/+}$ or *Foxc2*$^{lx/lx}$) or 28weeks (*Foxc2*$^{lx/+}$) was similar to littermate controls (Fig. 7D,E), demonstrating that EC Foxc2 expression is not required for VV maintenance.

Discussion

We have identified profound structural VV defects in patients carrying mutations in the genes encoding the transcription factor FOXC2*,* and the gap junction protein CX47. Crucially we have extended our understanding of VV development in mice to the earliest endothelial events and have identified a temporospatially regulated pattern of transcription factor and gap junction protein expression around Prox1^{hi} valve-forming cells (VFCs). This is required for the organisation of an initial ring of VFCs that is then critical for on-going VV development. Using a genetic loss-of-function approach, combined with drug inhibitors, we have demonstrated requirements for Cx37, Cx43 and Cx47 and provided evidence for Foxc2-Nfatc1 cooperative signaling in the regulation of VFC organisation at P0,. At later stages we show requirements for Foxc2, calcineurin-Nfat signalling and blood flow in regulating the maturation of valve leaflets.

Human VV phenotypes

Valve defects were particularly severe in patients with *FOXC2* mutations, with 75% fewer valves, and remaining valves were almost half the length of valves in matched controls. This finding is consistent with the increased incidence of chronic venous insufficiency that we have previously identified in this group of patients.(Brice, 2002; Mellor et al., 2007) Patients with mutations in *GJC2* had half the number of valves and those present were less than half the leaflet length of those seen in controls. Again, this is consistent with the reports of incompetent veins in these patients.(Ostergaard et al., 2011) The human *GJC2* mutations studied here produce an amino acid substitution in the first extracellular loop (which may affect connexon docking), and one in the intracellular loop domain.(Molica et al., 2014; Ostergaard et al., 2011) The mutation in *GJA1* (Cx43) results in a substitution (K206R) in the highly conserved SRPTEK motif of the second extracellular loop and may exert dominant negative effects on connexon docking, gap junction function and levels of wild-type protein.(Brice et al., 2013; Molica et al., 2014) The finding of fewer VVs in a single patient (albeit without statistical significance) is consistent with the VV developmental defects seen in mice after *Gja1* deletion, and requires exploration as further *GJA1*-mutated patients are identified.

Transcriptional regulation of murine VV formation

Our studies in mice extend previous descriptions of VV development, with our analysis of intact veins showing that initial events in VV formation occur earlier than we and others have previously identified.(Bazigou et al., 2011; Munger et al., 2016; Munger et al., 2013) We also show that valve development is affected by the background of the mouse strain used (Suppl. Fig 1).

In lymphatic endothelium, Foxc2 and Nfatc1 are co-expressed, physically interact, and signal co-operatively to pattern vessel maturation.(Norrmen et al., 2009) We examined proximal wholemount vein samples and found that Foxc2 and Nfatc1 were co-expressed by VFCs and endothelium downstream of the organizing VV. We report that whilst neither endothelial Foxc2 nor calcineurin-Nfat signaling alone are required for organisation of initial valve ring patterning, combined inhibition of Nfat signaling and *Foxc2*-deletion results in complete disorganisation of the valve-forming region.

This leads us to suggest that there is a degree of redundancy in regulation at this time point. It is difficult to fully exclude that a very low level of Foxc2 protein or calcineurin-Nfat signaling remained after *Foxc2/Ppp3r1* deletion and inhibition of Nfat signaling. However, we could find no Foxc2 immunosignal present in VVs 4days after induction of deletion (data not shown) in 4week-old mice, suggesting that Foxc2 depletion in VVs was efficient. In our experiments we either deleted *Ppp3r1* (CnB1) or administered CsA. Whilst we did not observe specific staining in developing VV using antibodies raised against Nfatc2, Nfatc3, or Nfatc4 (data not shown) we cannot fully exclude possible roles for these proteins.

Connexins pattern valve forming cell organisation

Whilst variation in connexin expression in veins at P0 has recently been reported, the expression was not related to positioning of sites of VV formation, which was not described until P4 when VV leaflets are already maturing.(Munger et al., 2016) Here, whole mount techniques enabled us to demonstrate that expression of Cx37, Cx43 and Cx47 are specifically temporo-spatially regulated relative to valve-forming cells (VFCs) at an earlier stage, between E18 and P0. Whilst Cx37 was expressed by Prox1^{hi} VFCs, Cx43 and Cx47 were predominantly expressed by cells upstream of the VFCs rather than by VFCs themselves. Despite this, loss of *Gja1*, *Gjc2* and *Gja4* all resulted in failure of organisation of the early Prox1^{hi} VFCs, characterised by loss of Prox1^{hi} elongation and reorientation. Cx37 loss resulted in a lower proportion of VFCs in the central portion of the vein, but this was not seen for loss of Cx43 or Cx47. Loss of Cx37 or Cx47 resulted in reduced VFC proliferation. It remains unknown whether this is a direct effect of the loss of gap junctions, or as a result of the failure of VFCs to organise normally, which could lead to reduced cell-cell signalling and loss of reinforcement of VFC identity. The loss of proliferation seen with *Gja4* knockout is surprising, as in other settings loss of Cx37 is associated with increased proliferation.(Burt et al.; Kanady et al.; Morel et al.) Whilst we did not detect apoptotic VFCs, we cannot exclude the possibility that any apoptotic cells were rapidly washed downstream. Taken together, our data would suggest that in the setting of VVs it may be a failure of Prox1^{hi} cell organisation (and resulting breakdown of inter-VFC communication) that results in the subsequent failure of further valve development in these mice.

Our TEM analysis of cell-cell junctions between free-edge (Prox1^{hi}) VFCs at P0 shows the presence of gap junctions. It is possible that groups of endothelial cells could develop discrete regions of gap junction intercellular communication, for example upstream of the initial Prox1^{hi} ring (Cx43, Cx47), or between free-edge cells themselves (Cx37), allowing for patterning of development by restricted regional endothelial signal propagation.(de Wit and Griffith, 2010; Pfenniger et al., 2012; Tallini et al., 2007) Regional variations in endothelial connexin expression have important roles in cardiac valve formation, for example patterning the transcriptional activity of Nfatc1 by Cx45 in endocardial cushion endothelial cells (where deletion of Cx45 abrogated Nfatc1 signaling).(Kumai et al., 2000) Sometimes connexins have roles entirely independent from intercellular communication. For example, Cx43 modulates cell polarity and directional cell migration (Francis et al., 2011). Whilst this cell-autonomous role could not entirely explain disorganisation of VFCs as we could find no Cx43 in the vast majority of these cells at P0, Cx43 loss could disrupt behaviour of

endothelial cells immediately upstream of the Prox1^{hi} cells. The population(s) of cells contributing to VV have not been defined and could include contributions from this upstream region, for example by becoming leaflet ECs, or by communicating with valve cells to regulate their behaviour. Interpretation of connexin phenotypes is further complicated by the formation of heteromeric and/or heterotypic channels.(Goodenough and Paul, 2009; Molica et al., 2014)

Patterning of valve position

Despite the Prox1^{hi} disorganisation phenotype seen with loss of *Gja1*, *Gjc2* and *Gja4*, VFCs were nonetheless located at the normal site of VV formation, and in a broad region across the vessel, suggesting that other factors are responsible for the positioning of the VV along the vein, initial VFC specification, and the overall patterning of this initial ring of VFCs. The finding of roles for *Gja1* and *Gja4* in patterning VFC reorientation and elongation at P0 is consistent with both the molecular similarity between VV and LV that we previously reported (Bazigou et al., 2011), and previous reports of absent VVs in *Gja4*$^{-/-}$ and *Gjc2*$^{-/-}$ mice identified at later ages (Munger et al., 2016; Munger et al., 2013).

Regulation of leaflet maturation and maintenance

Our data indicate differential requirements for maintenance of the free-edge cells during later development beyond P0. To more accurately assess the VV phenotype seen with loss of *Itgα9* and *Efnb2*, we carried out whole-vein confocal imaging of mice with postnatal deletion of *Itgα9* and *Efnb2* induced from P0.(Bazigou et al., 2011) As expected, at P6 a severe phenotype was seen in both mutants, with complete loss of VV leaflets, but with some residual Prox1^{hi} VFCs remaining (Suppl. Fig. 5A-C).(Bazigou et al., 2011) By comparison, valve cells were completely absent at P2 with loss of Cx47, and at P6 with loss of Cx37. This phenotype was more severe than that identified with loss of integrin-a9, ephrinb2 (when many Prox1^{hi} cells remained) or CnB1 (when a strikingly complete VFC ring remained). This restricts the requirement for Nfatc1 to extension of the VV leaflets and not maintenance of VFC identity. Our results suggest that Cx37 and Cx47 are required for the early stability of the VFC-ring phenotype, as has previously been suggested for Cx37 in LV.(Sabine et al., 2012) These results are also in accordance with a report of failed invagination of lymphovenous valves in *Gja4*$^{-/-}$ mice.(Geng et al.) Cx37 remains highly expressed by VV and may additionally be required for VV maintenance. In *Gjc2*$^{GFP/+}$ mice, Cx47-expressing cells were clearly identified at P6, suggesting that Cx47 could also have on-going roles in maintenance. Delineation of possible roles for Cx37 and Cx47 after P0 will require a conditional loss-of-function approach. The role of Cx43 was examined by conditional deletion in ECs and we found that this connexin was required for early but not later stages of valve development. *Gja4*$^{-/-}$ mice failed to develop an area of reduced SMA-expressing cells around the valve by P6, most likely secondary to loss of the valve ECs.(Bouvree et al., 2012; Jurisic et al., 2012)

Calcineurin-Nfat signaling was critical for leaflet elongation and commissure formation, consistent with its requirements for leaflet growth in aortic valve and LV.(Chang et al., 2004; Johnson, 2002; Sabine et al., 2012) Conditional homozygous deletion of *Foxc2* similarly produced a phenotype during leaflet maturation. In

contrast to the requirement for *Foxc2* in LV maintenance (Sabine et al., 2015), we found that *Foxc2* was not important for VV maintenance during the timeframe investigated. The timing of onset of VV failure in *FOXC2*-mutated patients is unknown; further work should explore whether VV in these patients develop normally and later regress, or fail to develop at all. We speculate that other transcription factors (for example FOXC1) could regulate VV and compensate for loss of FOXC2.(Fatima et al., 2016)

The role of blood flow

The regulators of Cx37, Cx43 and Cx47 in VV formation remain unclear. In vitro and in vivo experiments indicate that Foxc2 regulates expression of Cx37 in lymphatic endothelium.(Munger et al., 2013; Sabine et al., 2012) Whilst both are expressed by VFCs, the lack of VFC phenotype seen after *Foxc2* deletion suggests that other factors can compensate for loss of Foxc2 in these cells at P0. (Kanady et al., 2015; Munger et al., 2013) For example, Klf2 regulates Cx37 expression in blood endothelium in regions exposed to high laminar shear stress.(Pfenniger et al., 2012) Alternatively, induction of a GATA2/Foxc2/Prox1 pathway by oscillatory shear has been proposed as a mechanism for initiation of LV formation.(Kazenwadel et al.; Sabine et al., 2012; Sweet et al., 2015) GATA2 is expressed by venous VFCs (data not shown) and a similar process may regulate VV initiation. The early protrusion of VFCs into the lumen, and associated shear exposure, may provide a further mechanism to regulate expression in these cells. Our finding of smaller, less developed VVs, following reduction in blood flow through the femoral vein (albeit during VV maturation, not initiation) is consistent with this concept. We cannot confirm, however, that this effect is valve-specific rather than a general effect on endothelial cell growth.

Conclusions

Patients with mutations in *FOXC2* and *GJC2* have globally reduced numbers of VV and shorter VV leaflets. Foxc2 and Calcineurin-Nfatc1 signalling cooperate to organise the initial ring of VV-forming cells. Cx37, Cx43 and Cx47 are critical for early organisation of valve-forming cells at P0 and failure of this process likely underlies abnormal VV identified in patients with mutations in *GJC2*. Foxc2 expression in valve ECs is not required for VV maintenance.

Materials and Methods

All human and animal studies were performed in accordance with national regulations and ethical approvals (National Research Ethics Service Committee, South East Coast 10/H0701/68, 12/LO/1164, and UK Home Office)

Venous valve ultrasonography

The following veins underwent ultrasonographic evaluation: the brachial (ante-cubital fossa (ACF) to axillary vein; medial brachial vein if paired) basilic (ACF to axillary), popliteal (adductor hiatus to trifurcation), and short saphenous (sapheno-popliteal junction if within 10cm of knee skin-crease to 20cm below knee). Veins were visualised along their entire length, switching between longitudinal and transverse views to detect VV, using a Phillips IU22 with L17-5MHz/L9-3MHz probes. Participants with any history of deep vein thrombosis were excluded, as were any ablated/operated veins. Images and cine loops were recorded of each VV in B-mode and with colour Doppler. VV maximum leaflet measurements were obtained offline (Xcelera Cath Lab software, Phillips). For each vein, the number of VVs and VV length was normalised to the mean value in the respective control veins.

Human Genotyping

The screening of the *FOXC2* patients was performed by the South West Thames Regional Genetics Service at St George's, University of London. Screening for *GJC2* and *GJA1* was performed as previously described (Brice et al., 2013; Ostergaard et al., 2011)

Mouse Lines

Briefly, BALB/c, MF1, FVB, C57BL/6 and CD1 wild-type mice were obtained from Charles River UK. Wild-type analyses were performed in BALB/c mice unless indicated. *Prox1CreER^{T2}* (Bazigou et al., 2011), *Foxc2^{lx}* (Sasman et al., 2012), *Gja4$^{-/-}$* (Simon et al., 1997), *Gjc2^{GFP}*(Odermatt et al., 2003), *Gja1^{lx}(Calera et al., 2006), Ppp3r1^{lx}* (Zeng et al.), Rosa26^{mTmG} (Muzumdar et al., 2007) mice have been described previously and were maintained on C57BL/6 backgrounds, except *Prox1CreER^{T2};Foxc2^{lx}* which was back-crossed onto BALB/c. For the induction of Cre activity in *Prox1CreER^{T2}* mice, Tamoxifen/4OH-Tamoxifen (in sunflower oil, Sigma) was injected i.p. either 5mg at E15 for analysis at P0, or 50µg at P0 for analysis at P6.(Bazigou et al., 2011) 37.5µg/g.Ms weight progesterone was given i.p. at E15+E18 and embryos analysed at 'E19' (equivalent to P0). In all experiments we compared VV in *Prox1CreER^{T2}+* with *Prox1CreER^{T2}-* littermate controls. For drug inhibition of Nfat nuclear translocation 50µg/g.Ms weight Cyclosporin A (CsA, Calbiochem) was administered i.p. b.i.d. P1-P6 and VV analysed at P6. For combined deletion of *Foxc2* and calcineurin inhibition, deletion was performed from E15, and CsA 50µg/g.Ms weight i.p. b.d. from E17 to E19. For induction of deletion at 4 weeks, mice received 1mg Tam o.d. i.p. for 2days. For flow alteration at P0, pups were anaesthetized (Univentor 4000) with isofluorane and the femoral artery and vein (which are inseparable at P0) ligated (10/0 vicryl) and the vein divided prior to would closure (10/0 vicryl) and post-operative care with the mother.

Electron microscopy

For TEM, P0 pups were culled and perfused via the aorta with heparinised PBS (hPBS, 25mg/L MP Biomedicals) prior to fixation overnight in glutaraldehyde (2.5% v/v in 0.1M cacodylate buffer, pH 7.4, 4^{o}C) and post-fixation in osmium tetroxide (1% w/v in 0.1M cacodylate, pH 7.4, 4^{o}C) for 1.5hrs. For visualisation of murine gap junctions, samples were immersed in 1% osmium tetroxide: 1.125% potassium ferrocyanide for 1h followed by *en block* staining with 1% aqueous uranyl acetate. All samples were dehydrated through graded ethanols, equilibrated with propylene oxide, infiltrated with epoxy resin (TAAB) and polymerised at 70^{o}C for 24hrs. Semithin sections (0.5µm) were cut and stained with 1% Toluidine Blue. Ultrathin sections (50-70nm, Reichert-Jung ultramicrotome) were mounted and contrasted using uranyl acetate/lead citrate for examination (Hitachi H7600, 80kV, AMT digital camera).

For SEM, pups were culled and perfused via the IVC with heparinised PBS prior to opening the femoral and iliac veins and overnight fixation at 4^{o}C in glutaraldehyde (2.5% (v/v) in 0.1M cacodylate buffer, pH 7.4) and post-fixation in 1% (w/v) osmium tetroxide for 40mins at room temperature. After washing, samples were dehydrated through graded ethanols before critical point drying (Polaron E3000, Quorum Technologies) and mounting with conductive carbon cement (TAAB) on aluminium pins (TAAB) and gold sputter coating (Emitech K550X, Quorum Technologies) for examination (Hitachi S3500N, 20kV, high vacuum mode). VV leaflet length, position were measured in NIH ImageJ.

Immunohistochemistry

Human: For localization of connexins in human VVs (obtained from patients undergoing coronary artery bypass grafting) 10µm frozen sections were thawed and fixed in -20^{o}C MeOH prior to permeabilisation in 0.2% TritonX-100, charge neutralisation in 0.5M NH_4Cl, blocking in 2% BSA, and incubation with primary antibody. For localisation of FOXC2, after fixation in -20^{o}C acetone and quenching of endogenous peroxidase using 3%H_2O_2, blocking (X0909, DAKO), and incubation with primary antibody, signal was amplified using polymer-HRP (MP-XCP, Menarini) according to the manufacturer's instructions. Localisation of integrin-α9 was carried out on formalin-fixed paraffin embedded sections, using tyramide amplification (Perkin Elmer) according to the manufacturer's instructions.
Mouse: Mice were culled and perfusion fixed via the aorta and femoral vein with hPBS followed by fixation with 4% formaldehyde, and then further fixed for 24hours. The external iliac and femoral veins were excised with surrounding muscles and processed for paraffin wax embedding and Haematoxylin and Eosin stains. 5µm sections were photographed using a Micropublisher 3.3RTV camera mounted on a Leitz DMRB microscope with PL Fluotar ×10, ×20, and ×40 lenses (Leica). Maximum leaflet length of each valve was measured in NIH ImageJ.

Wholemount immunostaining

Mice were culled and perfused with hPBS via the aorta prior to fixation in 4% paraformaldehyde (PFA) followed by blocking in 3%v/v donkey serum in 0.3% Triton-x100. Samples were further dissected prior to incubation with primary antibodies, and washed prior to localisation with fluorophore-conjugated secondary antibodies. For co-localisation of Nfatc1 and Foxc2, samples were blocked with Fab (donkey anti goat IgG H+L, 100µg/ml, Jackson Immunoresearch) between sequential incubation with antibodies raised against Nfatc1 followed by Foxc2. Samples were then further dissected and mounted in Prolong Gold (Invitrogen). The consistent site of VV formation in unopened murine femoral vein was visualised by confocal microscopy (Leica SP5) to produce Z projections (NIH ImageJ) of median filtered (Leica LASAF/ImageJ, except for connexin localisation) stacks. VV Prox1^{hi} nuclear elongation (length:width ratio) and rotation (relative to the long axis of the vessel) were quantified in z-projections in ImageJ as previously described.(Tatin et al., 2013) Rotation was analysed in nuclei with length:width ratio ≥2. The position of each nucleus was taken as the center of the length measurement, and nuclear centile position across the vein was calculated (Microsoft Excel) and used to allocate nuclei into tertiles according to position across the vein. For analysis of proliferating VFCs, Prox1^{hi};Ki67^{+} nuclei were counted in alternate 1.5µm optical sections (NIH ImageJ) and presented as a proportion of total Prox1^{hi} VFCs. A reduced z-projection (4 slices, 6µm) is shown (Fig. 5F).

Antibodies

Immunohistochemistry: - antibodies were raised in rabbit to CX43 (Cell Signalling 3512), sheep to FOXC2 (AF5044, R&D), Foxc2 (AF6989, R&D) and goat to Integrinα9 (BAF3827, R&D)
Wholemount: antibodies were raised in rabbit to Cx43 (Cell Signalling 3512), Cx37 (CX37A11, Alpha Diagnostics International), Prox1 (11-002P, Angiobio), Cleaved Caspase 3 (Cell Signalling 9661), Ki67 (ab15580, Abcam), sheep to Foxc2 (AF6989, R&D), goat to Nfatc1 (AF5640, R&D), Prox1 (AF2727, R&D) and GFP (ab6658, Abcam), rat to PECAM1 (BD clone MEC 13.3) and mouse to alpha smooth muscle actin (clone 1A4 conjugated to Cy3, Sigma or FITC, Abcam).

Signal detection: - Secondary antibodies or streptavidin were conjugated to Dylight-405/488/550/649 (Jackson Immunoresearch) and streptavidin-HRP/alkaline phosphatase (DAKO). IgG controls were non-immune sheep/rabbit/goat IgG (R&D).

Statistical analysis

For human ultrasound, age and sex matching was tested respectively by ANOVA (with Bonferroni) and Fisher's exact test. All comparisons of human VV disease phenotypes were carried out using ANOVA with Bonferroni correction. In mice, for VV developmental stage 0-4 quantification, data represent the proportion of analysed valves reaching each developmental stage. P values represent difference in proportion of valves at each stage versus wild-type littermates (Chi square/Fisher's Exact). For quantification of connexin deletion phenotypes (Fig. 5) the proportion of nuclei

elongated ≥2, and for elongated nuclei, rotated ≥40degrees, was compared using chi-square tests as previously described.(Tatin et al., 2013)

For analysis of *CnB1* deleted VV (Fig. 6), leaflet length was compared using a standard template in z-projections (independent samples t-test). VV area was compared using an independent samples t-test. Abnormal VV development after CsA administration to P6 was compared by Chi Square test. For VV maintenance, independent samples t test was used for 2 groups, and ANOVA for three groups. $P<0.05$ was considered significant. All analyses were carried out using IBM SPSS v22.

Supplemental Material

Further details including patient characteristics, VV variability in murine strains, and individual channels for multi-channel images are provided in supplementary material.

References

Bazigou, E., O.T. Lyons, A. Smith, G.E. Venn, C. Cope, N.A. Brown, and T. Makinen. 2011. Genes regulating lymphangiogenesis control venous valve formation and maintenance in mice. *J Clin Invest* 121:2984-2992.

Bergan, J.J., G.W. Schmid-Schonbein, P.D. Smith, A.N. Nicolaides, M.R. Boisseau, and B. Eklof. 2006. Chronic venous disease. *N Engl J Med* 355:488-498.

Bouvree, K., I. Brunet, R. Del Toro, E. Gordon, C. Prahst, B. Cristofaro, T. Mathivet, Y. Xu, J. Soueid, V. Fortuna, N. Miura, M.S. Aigrot, C.H. Maden, C. Ruhrberg, J.L. Thomas, and A. Eichmann. 2012. Semaphorin3A, Neuropilin-1, and PlexinA1 are required for lymphatic valve formation. *Circ Res* 111:437-445.

Brice, G. 2002. Analysis of the phenotypic abnormalities in lymphoedema-distichiasis syndrome in 74 patients with FOXC2 mutations or linkage to 16q24. *Journal of Medical Genetics* 39:478-483.

Brice, G., P. Ostergaard, S. Jeffery, K. Gordon, P. Mortimer, and S. Mansour. 2013. A novel mutation in GJA1 causing oculodentodigital syndrome and primary lymphoedema in a three generation family. *Clin Genet*

Burt, J.M., T.K. Nelson, A.M. Simon, and J.S. Fang. 2008. Connexin 37 profoundly slows cell cycle progression in rat insulinoma cells. *American journal of physiology. Cell physiology* 295:C1103-1112.

Caggiati, A.C., L. 2013. Surgery of venous valve. *Reviews in vascular medicine* 1:15-23.

Calera, M.R., H.L. Topley, Y. Liao, B.R. Duling, D.L. Paul, and D.A. Goodenough. 2006. Connexin43 is required for production of the aqueous humor in the murine eye. *J Cell Sci* 119:4510-4519.

Chang, C.-P., J.R. Neilson, J.H. Bayle, J.E. Gestwicki, A. Kuo, K. Stankunas, I.A. Graef, and G.R. Crabtree. 2004. A Field of Myocardial-Endocardial NFAT Signaling Underlies Heart Valve Morphogenesis. *Cell* 118:649-663.

Czarniawska-Grzesinska. 2002. Development of valves in the small saphenous vein in human fetuses. *Folia Morphol* 61:37-42.

de la Pompa, J.L., L.A. Timmerman, H. Takimoto, H. Yoshida, A.J. Elia, E. Samper, J. Potter, A. Wakeham, L. Marengere, B.L. Langille, G.R. Crabtree, and T.W. Mak. 1998. Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature* 392:182-186.

de Wit, C., and T.M. Griffith. 2010. Connexins and gap junctions in the EDHF phenomenon and conducted vasomotor responses. *Pflugers Archiv : European journal of physiology* 459:897-914.

Eberhardt, R.T., and J.D. Raffetto. 2014. Chronic venous insufficiency. *Circulation* 130:333-346.

Fang, J., S.L. Dagenais, R.P. Erickson, M.F. Arlt, M.W. Glynn, J.L. Gorski, L.H. Seaver, and T.W. Glover. 2000. Mutations in FOXC2 (MFH-1), a forkhead family transcription factor, are responsible for the hereditary lymphedema-distichiasis syndrome. *Am J Hum Genet* 67:1382-1388.

Fatima, A., Y. Wang, Y. Uchida, P. Norden, T. Liu, A. Culver, W.H. Dietz, F. Culver, M. Millay, Y.S. Mukouyama, and T. Kume. 2016. Foxc1 and Foxc2 deletion causes abnormal lymphangiogenesis and correlates with ERK hyperactivation. *J Clin Invest*

Ferrell, R.E., C.J. Baty, M.A. Kimak, J.M. Karlsson, E.C. Lawrence, M. Franke-Snyder, S.D. Meriney, E. Feingold, and D.N. Finegold. 2010. GJC2 missense mutations cause human lymphedema. *Am J Hum Genet* 86:943-948.

Francis, R., X. Xu, H. Park, C.J. Wei, S. Chang, B. Chatterjee, and C. Lo. 2011. Connexin43 modulates cell polarity and directional cell migration by regulating microtubule dynamics. *PLoS One* 6:e26379.

Geng, X., B. Cha, M.R. Mahamud, K.C. Lim, R. Silasi-Mansat, M.K. Uddin, N. Miura, L. Xia, A.M. Simon, J.D. Engel, H. Chen, F. Lupu, and R.S. Srinivasan. 2016. Multiple mouse models of primary lymphedema exhibit distinct defects in lymphovenous valve development. *Dev Biol* 409:218-233.

Goodenough, D.A., and D.L. Paul. 2009. Gap junctions. *Cold Spring Harbor perspectives in biology* 1:a002576.

Iimura, A., Y. Nakamura, and M. Itoh. 2003. Anatomical study of distribution of valves of the cutaneous veins of adult's limbs. *Annals of anatomy = Anatomischer Anzeiger : official organ of the Anatomische Gesellschaft* 185:91-95.

Johnson, E.N. 2002. NFATc1 Mediates Vascular Endothelial Growth Factor-induced Proliferation of Human Pulmonary Valve Endothelial Cells. *Journal of Biological Chemistry* 278:1686-1692.

Jurisic, G., H. Maby-El Hajjami, S. Karaman, A.M. Ochsenbein, A. Alitalo, S.S. Siddiqui, C. Ochoa Pereira, T.V. Petrova, and M. Detmar. 2012. An unexpected role of semaphorin3a-neuropilin-1 signaling in lymphatic vessel maturation and valve formation. *Circ Res* 111:426-436.

Kanady, J.D., M.T. Dellinger, S.J. Munger, M.H. Witte, and A.M. Simon. 2011. Connexin37 and Connexin43 deficiencies in mice disrupt lymphatic valve development and result in lymphatic disorders including lymphedema and chylothorax. *Dev Biol* 354:253-266.

Kanady, J.D., S.J. Munger, M.H. Witte, and A.M. Simon. 2015. Combining Foxc2 and Connexin37 deletions in mice leads to severe defects in lymphatic vascular growth and remodeling. *Dev Biol* 405:33-46.

Kazenwadel, J., K.L. Betterman, C.E. Chong, P.H. Stokes, Y.K. Lee, G.A. Secker, Y. Agalarov, C.S. Demir, D.M. Lawrence, D.L. Sutton, S.P. Tabruyn, N. Miura, M. Salminen, T.V. Petrova, J.M. Matthews, C.N. Hahn, H.S. Scott, and N.L. Harvey. 2015. GATA2 is required for lymphatic vessel valve development and maintenance. *J Clin Invest* 125:2979-2994.

Kumai, M., K. Nishii, K. Nakamura, N. Takeda, M. Suzuki, and Y. Shibata. 2000. Loss of connexin45 causes a cushion defect in early cardiogenesis. *Development* 127:3501-3512.

Lane, R.G., JA. Cuzzila, ML. Coroneoa, JC. 2007. Incompetent venous valves: Ultrasound imaging and exo-stent repair. . *Phlebolymphology* 14:105-115.

Lurie, F., and R.L. Kistner. 2012. The relative position of paired valves at venous junctions suggests their role in modulating three-dimensional flow pattern in veins. *Eur J Vasc Endovasc Surg* 44:337-340.

Maleti, O., M. Lugli, and R.K. Tripathi. 2015. Deep venous reconstructive surgery. *Semin Vasc Surg* 28:39-46.

May, D., O. Tress, G. Seifert, and K. Willecke. 2013. Connexin47 protein phosphorylation and stability in oligodendrocytes depend on expression of Connexin43 protein in astrocytes. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 33:7985-7996.

Meissner, M., G. Moneta, K. Burnand, P. Gloviczki, J. Lohr, F. Lurie, M. Mattos, R. McLafferty, G. Mozes, and R. Rutherford. 2007. The hemodynamics and diagnosis of venous disease. *Journal of Vascular Surgery* 46:S4-S24.

Mellor, R.H., G. Brice, A.W.B. Stanton, J. French, A. Smith, S. Jeffery, J.R. Levick, K.G. Burnand, and P.S. Mortimer. 2007. Mutations in FOXC2 Are Strongly Associated With Primary Valve Failure in Veins of the Lower Limb. *Circulation* 115:1912-1920.

Molica, F., M.J. Meens, S. Morel, and B.R. Kwak. 2014. Mutations in cardiovascular connexin genes. *Biol Cell* 106:269-293.

Moore, H.M., M. Gohel, and A.H. Davies. 2011. Number and location of venous valves within the popliteal and femoral veins: a review of the literature. *Journal of anatomy* 219:439-443.

Morel, S., L. Burnier, A. Roatti, A. Chassot, I. Roth, E. Sutter, K. Galan, A. Pfenniger, M. Chanson, and B.R. Kwak. 2010. Unexpected role for the human Cx37 C1019T polymorphism in tumour cell proliferation. *Carcinogenesis* 31:1922-1931.

Munger, S.J., X. Geng, R.S. Srinivasan, M.H. Witte, D.L. Paul, and A.M. Simon. 2016. Segregated Foxc2, NFATc1 and Connexin expression at normal developing venous valves, and Connexin-specific differences in the valve phenotypes of Cx37, Cx43, and Cx47 knockout mice. *Dev Biol* 412:173-190.

Munger, S.J., J.D. Kanady, and A.M. Simon. 2013. Absence of venous valves in mice lacking Connexin37. *Dev Biol* 373:338-348.

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

Norrmen, C., K.I. Ivanov, J. Cheng, N. Zangger, M. Delorenzi, M. Jaquet, N. Miura, P. Puolakkainen, V. Horsley, J. Hu, H.G. Augustin, S. Yla-Herttuala, K. Alitalo, and T.V. Petrova. 2009. FOXC2 controls formation and maturation of lymphatic collecting vessels through cooperation with NFATc1. *The Journal of Cell Biology* 185:439-457.

Odermatt, B., K. Wellershaus, A. Wallraff, G. Seifert, J. Degen, C. Euwens, B. Fuss, H. Bussow, K. Schilling, C. Steinhauser, and K. Willecke. 2003. Connexin 47 (Cx47)-deficient mice with enhanced green fluorescent protein reporter gene reveal predominant oligodendrocytic expression of Cx47 and display vacuolized myelin in the CNS. *The Journal of neuroscience : the official journal of the Society for Neuroscience* 23:4549-4559.

Ostergaard, P., M.A. Simpson, G. Brice, S. Mansour, F.C. Connell, A. Onoufriadis, A.H. Child, J. Hwang, K. Kalidas, P.S. Mortimer, R. Trembath, and S. Jeffery. 2011. Rapid identification of mutations in GJC2 in primary lymphoedema using whole exome sequencing combined with linkage analysis with delineation of the phenotype. *J Med Genet* 48:251-255.

Petrova, T.V., T. Karpanen, C. Norrmén, R. Mellor, T. Tamakoshi, D. Finegold, R. Ferrell, D. Kerjaschki, P. Mortimer, S. Ylä-Herttuala, N. Miura, and K. Alitalo. 2004. Defective valves and abnormal mural cell recruitment underlie lymphatic vascular failure in lymphedema distichiasis. *Nature Medicine* 10:974-981.

Pfenniger, A., C. Wong, E. Sutter, S. Cuhlmann, S. Dunoyer-Geindre, F. Mach, A.J. Horrevoets, P.C. Evans, R. Krams, and B.R. Kwak. 2012. Shear stress modulates the expression of the atheroprotective protein Cx37 in endothelial cells. *Journal of molecular and cellular cardiology* 53:299-309.

Phillips, M.N., G.T. Jones, A.M. van Rij, and M. Zhang. 2004. Micro-venous valves in the superficial veins of the human lower limb. *Clin Anat* 17:55-60.

Rosbotham, J.L., G.W. Brice, A.H. Child, T.O. Nunan, P.S. Mortimer, and K.G. Burnand. 2000. Distichiasis-lymphoedema: clinical features, venous function and lymphoscintigraphy. *The British journal of dermatology* 142:148-152.

Sabine, A., Y. Agalarov, H. Maby-El Hajjami, M. Jaquet, R. Hagerling, C. Pollmann, D. Bebber, A. Pfenniger, N. Miura, O. Dormond, J.M. Calmes, R.H. Adams, T. Makinen, F. Kiefer, B.R. Kwak, and T.V. Petrova. 2012. Mechanotransduction, PROX1, and FOXC2 cooperate to control connexin37 and calcineurin during lymphatic-valve formation. *Dev Cell* 22:430-445.

Sabine, A., E. Bovay, C.S. Demir, W. Kimura, M. Jaquet, Y. Agalarov, N. Zangger, J.P. Scallan, W. Graber, E. Gulpinar, B.R. Kwak, T. Makinen, I. Martinez-Corral, S. Ortega, M. Delorenzi, F. Kiefer, M.J. Davis, V. Djonov, N. Miura, and T.V. Petrova. 2015. FOXC2 and fluid shear stress stabilize postnatal lymphatic vasculature. *J Clin Invest* 125:3861-3877.

Sasman, A., C. Nassano-Miller, K.S. Shim, H.Y. Koo, T. Liu, K.M. Schultz, M. Millay, A. Nanano, M. Kang, T. Suzuki, and T. Kume. 2012. Generation of conditional alleles for Foxc1 and Foxc2 in mice. *genesis* 50:766-774.

Schweighofer, G., D. Muhlberger, and E. Brenner. 2010. The anatomy of the small saphenous vein: fascial and neural relations, saphenofemoral junction, and valves. *J Vasc Surg* 51:982-989.

Simon, A.M., D.A. Goodenough, E. Li, and D.L. Paul. 1997. Female infertility in mice lacking connexin 37. *Nature* 385:525-529.

Srinivasan.RS, O.G. 2011. Prox1 dosage controls the number of lymphatic endothelial cell progenitors and the formation of the lymphovenous valves. *Genes Dev* 25:2187-2197.

Sweet, D.T., J.M. Jimenez, J. Chang, P.R. Hess, P. Mericko-Ishizuka, J. Fu, L. Xia, P.F. Davies, and M.L. Kahn. 2015. Lymph flow regulates collecting lymphatic vessel maturation in vivo. *J Clin Invest* 125:2995-3007.

Tallini, Y.N., J.F. Brekke, B. Shui, R. Doran, S.M. Hwang, J. Nakai, G. Salama, S.S. Segal, and M.I. Kotlikoff. 2007. Propagated endothelial Ca2+ waves and arteriolar dilation in vivo: measurements in Cx40BAC GCaMP2 transgenic mice. *Circ Res* 101:1300-1309.

Tatin, F., A. Taddei, A. Weston, E. Fuchs, D. Devenport, F. Tissir, and T. Makinen. 2013. Planar Cell Polarity Protein Celsr1 Regulates Endothelial Adherens Junctions and Directed Cell Rearrangements during Valve Morphogenesis. *Dev Cell* 26:31-44.

Winnier, G.E., T. Kume, K. Deng, R. Rogers, J. Bundy, C. Raines, M.A. Walter, B.L. Hogan, and S.J. Conway. 1999. Roles for the winged helix transcription factors MF1 and MFH1 in cardiovascular development revealed by nonallelic noncomplementation of null alleles. *Dev Biol* 213:418-431.

Zeng, H., S. Chattarji, M. Barbarosie, L. Rondi-Reig, B.D. Philpot, T. Miyakawa, M.F. Bear, and S. Tonegawa. 2001. Forebrain-specific calcineurin knockout selectively impairs bidirectional synaptic plasticity and working/episodic-like memory. *Cell* 107:617-629.

Table 1 Mean number of valves in controls and patients

N VV per vein (normalised to control veins)			
	N veins analysed	Mean N VV per vein	SD
Control	68	1.00	0.53
FOXC2	64	0.25 ***	0.32
GJC2	24	0.38 ***	0.46
GJA1	8	0.63	0.41

*** P<0.0005 for FOXC2 and GJC2, for mean N valves (ANOVA, Bonferroni). Not all veins were scanned in all controls/patients.

Table 2 Mean VV leaflet length in controls and patients

Leaflet lengths (normalised to control veins)			
	N VV measured	Mean leaflet length (mm)	SD
Control	118	1.00	0.38
FOXC2	32	0.44 ***	0.25
GJC2	21	0.61 ***	0.28
GJA1	10	0.95	0.40

*** P<0.0005 for FOXC2 and GJC2, for mean leaflet length (ANOVA, Bonferroni)

Supplementary data

Supplementary Table 1: Characteristics of study participants

Characteristics of study participants. Participants were matched to the control group by age (P=NS) and sex (P=NS). Controls included non-mutation carrying relatives of affected individuals.

	ID	Age	Sex	Genotype	Effect
Control	1	24	F		
	2	24	M		
	3	25	M		
	4	30	F		
	5	37	M		
	6	42	F		
	7	42	F		
	8	53	F	Unaffected relatives	
	9	58	F		
	10	69	M		
FOXC2	11	20	M	c.223T>G p.(Tyr75Asp)	Substitution in forkhead domain
	12	47	M		
	13	21	M	c.438G>A p.(Trp146Ter)	Stop in forkhead domain (after 145aa)
	14	54	M		
	15	22	F	c.595dupC p.(His199Profs*264)	Nonsense from aa199 onwards
	16	31	F	c.361C>T p.(Arg121Cys)	Substitution in forkhead domain
	17	42	F	c.298C>T p.(Gln100Ter)	Stop in forkhead domain (after 99aa)
	18	51	M	c.595dupC p.(His199Profs*264)	Nonsense from aa199 onwards
GJC2	19	17	F	c.143C>T p.(Ser48Leu)	Substitution in 1st extracellular loop (Molica et al., 2014; Ostergaard et al., 2011)
	20	46	M		
	21	71	M	c.629T>G p.(Met210Arg)	Mutation in intracellular loop (Ostergaard et al., 2011)
GJA1	22	44	F	c.617A>G p.(Lys206Arg)	Substitution in conserved SRPTEK sequence(Brice et al., 2013)

Supplementary Table 2: Number of valves per vein in control group

	N valves per vein in controls			
	N veins	Mean N valves	SD	N VV analysed
Popliteal	16	1.13	0.72	18
Short saphenous	16	3.38	1.45	54
Brachial	18	1.83	1.20	33
Basilic	18	2.39	0.92	43

In control human veins, the number of VV detected and VV leaflet length followed approximately normal distributions; mean N VV and VV length varied significantly by vein ($P<0.0005$ ANOVA) and results were subsequently normalised to controls for each vein. SD=standard deviation

Supplementary Table 3: Leaflet lengths per vein in control group

	Leaflet length in controls		
	N VV measured	Mean VV length (mm)	SD
Popliteal	14	6.79	1.87
Short saphenous	40	4.00	1.36
Brachial	26	3.88	1.56
Basilic	38	4.93	2.18

Figure Legends

Figure 1: Human VV phenotypes

A) Venous valves (VV, arrowheads) were visualised in controls and patients carrying mutations in the indicated genes, without (left) and with (right) power doppler imaging of blood flow during a calf squeeze. □ marks stagnant/reversed flow in a VV sinus. Blood flow is from left to right. Scale 1mm B-C) Number (B) and length (C) of VV identified in patients carrying mutations in *FOXC2* (N=8 patients), *GJC2* (N=3), GJA1 (N=1) and controls (N=10). See Suppl. Tables 1-3. ***P<0.0005 (ANOVA with Bonferroni). Error bars represent mean ±SEM. D) Immunolocalisation (arrowheads) of FOXC2 and CX43 in adult human VV leaflets. Scale 20µm.

Figure 2: VV development in the proximal femoral vein

A-B) Micrographs before (A) and after (B) dissection to perform wholemount imaging of the proximal femoral vein (FV) valve (boxed region). IVC= Inferior vena cava, FA= femoral artery. Scale 200µm. C) Localisation of Prox1 (red), Foxc2 (green) and PECAM1 (blue) at the indicated time points. □ marks single commissure at Stage 3, and two commissures at Stage 4. Arrowheads= reorientated and elongated VFCs. Scale 20µm. N≥6 VV/stage. D) Corresponding schematics of stages in VV development: Prox1^{hi} free-edge cells (red), leaflets (green), and leaflet attachment to the vein wall (blue). Arrows= blood flow. E) A peri-VV reduction (between dotted lines) in SMA-expressing cells (Red) Scale 20µm. N>6. F) Quantification of elongated Prox1^{hi} valve cells on the anterior and posterior vein wall at P0. (N=9, Paired t test). G) Arrowheads= anterior wall VFCs. Scale 20µm. Blood flow left to right in C-G.

Figure 3: Regulation of initial VV organisation by Foxc2 and Nfatc1

A) Co-localisation of Foxc2 (red) and Nfatc1 (green) in VFCs at P0. Foxc2 and Nfatc1 localisation are also shown separately. N=4. Scale 20µm. B-E) The proportion of VV identified at Stage 0 (white) and Stage 1 (grey) at P0 after deletion of *Foxc2* (B) or *Ppp3r1* (CnB1, C) from E15, or treatment with ciclosporin from E17 (D), or with *combined Foxc2* deletion and ciclosporin treatment (E). N VV analysed for each condition is indicated above each bar. ***P<0.0005, Chi Square. In (E), localisation of Prox1 (red) is shown at P0 after combined *Foxc2* deletion and ciclosporin treatment. Scale 20µm

Figure 4: Connexins pattern valve organisation at P0

(A-F) Expression in wildtype mice (A,C,E) and the knockout or conditional deletion loss of function phenotypes at P0 for *Gja4*, *Gja1* and *Gjc2* (B,D,F). A) Immunolocalisation of Cx37 (arrowheads), and (B) of Prox1^{hi} VFCs (arrowheads) in Gja4$^{-/-}$ mice. C) Immunolocalisation of Cx43 (arrowheads), and (D) of Prox1^{hi} VFCs (arrowhead) in *Prox1CreER^{T2};Gja1$^{lx/lx}$* mice after induction of deletion at E15. E) Immunolocalisation of GFP (green) in *Gjc2$^{GFP/+}$* reporter mice, and (F) of Prox1^{hi} VFCs (arrowheads) in *Gjc2$^{GFP/GFP}$* knockout mice in. N≥6 for all images. Scale 20µm. Blood flow left to right throughout. Graphs in B,D and F show the proportion of VV identified at Stage 0 (white) and Stage 1 (grey) at P0 for the indicated genotypes, and N VVs analysed for each condition are given above each bar. *P<0.05, **P<0.005 Chi Square/Fisher's Exact. (G) shows a tiled TEM image of the junction between two adjacent free-edge cells at P0, shown at lower magnification in Suppl. Fig 3K. N=3. Scale 100nm, 10nm in inset.

Figure 5: Quantification of connexin phenotypes

(A) The schematic indicates how position and orientation of Prox1^{hi} VFCs were measured in A'-A''. A') Binned scatterplot of VFC elongation (length:width ratio) across the vein from superior (Tertile 3) to inferior (Tertile 1). N=1388 cells, 17 VV. The red dotted box indicates elongated nuclei (length:width ratio≥2), which were further analysed in A''. A'') Binned scatterplot of elongated VFC nuclear reorientation across the vein. The blue dotted box indicates nuclei rotated ≥40^{o}. N=953 cells, 17 VV. (B) The proportion of Prox1^{hi} VFC's in the middle tertile is shown for the indicated genotypes.**P<0.005, T Test. C) The elongation of Prox1^{hi} nuclei from the central tertile of valves of the indicated genotypes are shown. Elongated nuclei (length:width ratio≥2) are highlighted in red. D) The reorientation of elongated Prox1^{hi} nuclei from the central tertile of valves of the indicated genotypes are shown. Reorientated nuclei (≥40^{o}) are highlighted in blue. *P<0.05, **P<0.005, Chi Square. In B-D, N= 6 WT Vs 8 *Gja4$^{-/-}$* VV, 6 WT Vs 10 *Gja1$^{lx/lx}$* VV, 5 WT Vs 5 *Gjc2$^{-/-}$* VV. Data for all genotypes and vein regions provided in Suppl. Fig 4. E-F) Graphs indicate the mean number (as fold over wildtype littermate VV) of Prox1^{hi};Ki67^{+} proliferating VFCs for the indicated genotypes. N = 5 WT Vs 6 *Gja4$^{-/-}$* VV and 6 WT Vs 6 *Gjc2$^{-/-}$* VV. **P<0.005, ***P<0.0005, T Test. (F) Representative images are shown of Prox1 (red) and Ki67 (green) immunolocalisation in the upper region of the valve in *Gja4$^{-/-}$*, *Gjc2$^{-/-}$* mice and a WT littermate. Arrowheads indicate Prox1 and Ki67 co-expressing cells. Scale 10µm. Images are a 6µm-thick z-projection of 0.5µm optical sections.

Figure 6: Maturation of leaflets and commissures

A) Immunolocalisation at P6 of Nfatc1 (green) and Prox1 (red) in VV of WT mice administered control solvent or ciclosporin (N=5 Vs 8 VV, P<0.05, Chi Square). B) Immunolocalisation of Foxc2 (green) and Prox1 (red) in VV of mice of the indicated genotypes. N≥6 per condition. C, D) Quantification of leaflet length and stage of VV development in mice of the indicated genotypes. Error bars represent SEM. T test for leaflet length. In C,D,G,H and I, colours represent developmental stage (see key), N VVs analysed for each condition are given above each bar, and *P<0.05, **P<0.005 (Chi Square for developmental stage). E) Immunolocalisation of Cx37 (arrowheads) and Cx43 in VV leaflets at P6. N≥4. F) Localisation of Prox1, Foxc2 and SMA at P6 in *Gja4*$^{-/-}$ vein. N≥6 G,H) Stages of VV development reached in mice of the indicated genotypes. I) Localisation of Prox1, GFP and SMA in *Gjc2*$^{GFP/GFP}$ knockout reporter vein at P2 (N=4), and stages of VV development reached in mice of the indicated genotypes. J) Localisation of Cx47-expressing VV cells (arrowheads) of *Gjc2*$^{GFP/+}$ reporter at P6. N=4. Multichannel images are reproduced in supplementary data. Scale 20μm. Blue stain in A-J is PECAM1.

Figure 7: Role of blood flow in valve growth, and the regulation of VV maintenance

A-B) Representative images at P0 and P6 after single FV ligation (arrowheads in A) or double ligation and FV division (arrowheads in B) at P0, and quantification of the proportion of valves reaching each developmental stage at P6 in VV of operated and unoperated limbs. N VV analysed given above bars. □ indicates collateral vessel. Scale =500µm. **P<0.005, Chi Square. Dotted boxes X and Y indicate regions examined by immunofluorescence at P6. In X, arrowhead indicates suture, and arrow the remodeling femoral vein. In Y, □ indicates large collaterals and arrow the proximal FV. Scale 20µm in micrographs. C) Localisation of Foxc2 (green) and quantification of VV area in VV exposed to reduced flow (N=8) and controls (N=12). ***P<.0005, T test. Scale=20µm. D-E) Analysis of VV length (H&E) 2weeks (D) and 24weeks (E) after *Foxc2* deletion. P=NS (ANOVA). N VV analysed are given below each bar.

Supplementary figure legends

Supplementary figure 1: Variability in valves in different strains of mice

A-E) We noted variant VV in some wildtype C57BL6/J mice, and compared VV in several strains. A) Representative images (N≥6 per condition, as indicated in C) of VV in wildtype mice from the indicated strains at P11, indicating the variant of separated commissures around an unusually proximal tributary commonly seen in FVB (N=14 VV), C57BL6/J (N=33) and CD1 (N=14) strains but rarely in MF1 (N=16) and not in BALB/c (N=19). The pink dotted line indicates where leaflets have been cut during opening of the vein, and the blue dotted line marks the junction of the leaflet with the vein wall. Arrowheads indicate commissures (BALB/c, MF1) or abnormally separated commissures (FVB, C57BL6/J, CD1). The origin of the tributary normally just downstream of the valve is marked □, and lies between the separated commissures in images from FVB, C57BL6/J, CD1. Scale 100µm. Blood flow is upwards. B) Schematic of VV measurements obtained overlaid on a P16 left-sided VV. The distance between the commissures (blue line), distance from the center of the tributary to the most proximal commissure (perpendicular to the vessel centerline, red line), and maximum leaflet length (black line) was measured. Tributaries are marked with □, and some contain ostial valves. The region shown encompasses the proximal FV and external iliac vein and the VV analysed here lies in the proximal FV. Scale 100µm C) Number of VV with and without separated commissures in the strains indicated. *P<0.05, **P<0.005, Fisher's Exact for proportions. D-E) Leaflet length (D) and distance from tributary to proximal commissure (E) in SEM images, in the indicated strains, at P6 (green) and P11 (blue). Each marker represents one valve analysed. **P<0.005, ***P<0.0005. ANOVA with Bonferroni Post Hoc Vs BALB/C (at P11).

Supplementary figure 2: Regulation of initial VV organisation (Cn-Nfat signalling)

A) Representative images of immunolocalisation of Nfatc1 (green) and Prox1 (red) after *Ppp3r1* (CnB) deletion and in littermate controls. Arrowheads indicate (respectively) cytoplasmic and nuclear Nfatc1 localisation. N=4 per group. B-C) Cytoplasmic localisation (arrowheads) of Nfatc1 at E18 (C, N=5) and P0 (D) with CsA treatment. N=4 D) Control for the Foxc2/Nfatc1 colocalisation shown in Figure 3. Anti-Foxc2 primary antibody (raised in sheep) was omitted, demonstrating successful Fab block of cross-reactive binding of anti-sheep secondary to anti-Nfatc1 primary (raised in goat). N=3 Blue stain in A-D is PECAM1. Scale in A-D is 20µm.

Supplementary figure 3: Regulation of initial VV organisation

A-I) Multichannel confocal images are shown (along with the individual channels) for the genotypes, ages and proteins indicated. N≥6 per condition. Scale 20µm. FA=Femoral artery, FV= Femoral vein. B) Arrowheads indicate Cx37-expressing VFCs (reproduced from Fig. 4A). D) Arrowheads indicate disorganized VFCs. E) Arrowheads indicate predominant region of Cx43 localisation. F) Arrowheads indicate disorganized VFCs. G-I) □=GFP (*Gjc2*^{GFP}) expressing cells. The region of vein just upstream of G is shown in H. I) Arrowheads indicate VFCs. J) Tiled micrograph of a semi-thin section at P0 in which the FV VV (arrowhead) is easily identified as a projection into the lumen, upstream of a tributary. N=3. A major arterial branch runs perpendicular to the vein. The leading edge of the developing VV was examined by TEM (K), showing 3 leading-edge VFC's, with the dotted region shown in Fig. 4G (N=3). Scale 20µm in J, 200nm in K. N=3

Supplementary figure 4: Quantification of connexin phenotypes

A-B) Scatterplots are shown for VFC cellular Vs nuclear morphology (orientation in A, elongation in B) in double transgenic Prox1CreER^{T2};Rosa26^{mTmG} mice at P0, after induction of Cre at E15. N=47 cells. C) A Hex-binned scatterplot is shown for wildtype VFC nuclear reorientation and elongation. N= 1388 cells. D) Mean wildtype VFC nuclear reorientation is shown for each tertile, relating to Fig.5 A''. Error bars represent SEM. N=953 cells, ANOVA & Bonferroni. ***P<0.0005. E) Analysis of Prox1^{hi} VFC nuclear reorientation and elongation at P0 with loss of Gja4, Gja1 or Gjc2. Relates to Fig.5 C+D. Each data point represents one VFC. N= 6 WT Vs 4 *Gja4*$^{+/-}$ Vs 8 *Gja4*$^{-/-}$ VV, 6 WT Vs 10 *Gja1*$^{lx/lx}$ VV, and 5 WT Vs 6 *Gjc2*$^{+/-}$ Vs 5 *Gjc2*$^{-/-}$ VV.*P<0.05, **P<0.005, ***P<0.0005. Chi Square test (proportion elongated ≥2, or rotated ≥40degrees).

Supplementary figure 5: Maturation of leaflets and commissures

A-C) Following our change of method to earlier deletion and wholemount visualisation of closed veins, we confirmed the previously described phenotypes following deletion of *Efnb2* (A) and *Itga9* (B) using floxed alleles. N VV analysed given above bars. *P0.05, **P<0.005 Chi Square. C) Localisation of Foxc2 and tomato/GFP (mTmG line) is shown for the indicated genotypes at P6. Scale=20µm. D) Integrin-α9 was immunolocalised to human VV leaflets. Scale=50µm.

E-G) Multichannel confocal images are shown (along with the individual channels) for the genotypes, ages and proteins indicated. N≥6 per condition. Scale 20µm. E) Arrowheads indicate free edge cells). H) Arrowheads indicate Cx43, just detectable in VV leaflets (and lymphatics, □) at P2. J-M) Multichannel confocal images are shown (along with the individual channels) for *Gjc2* expression in the genotypes, ages and proteins indicated. *Gjc2* expression was examined using the $Gjc2^{GFP}$ reporter with amplification using antibodies raised against GFP. J) N=4, K) N=3, L) N=13, M) N=4 VV.