# MORPHOGENESIS OF LYMPHATIC VASCULAR NETWORKS: INSIGHTS FROM CONNEXIN AND FOXC2 KNOCKOUT MICE

by

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As members of the Dissertation Committee, we certify that we have read the dissertation prepared by John Kanady, titled *Morphogenesis of Lymphatic Vascular Networks: Insights from Connexin and Foxc2 Knockout Mice* and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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SIGNED: John Kanady

## ACKNOWLEDGEMENTS

I would like to thank my mentor, Alexander Simon, for taking the chance on a fledgling graduate student and allowing me to join his lab. While there were high times and low times, they have been the best years of my life. I learned more than I had hoped from his tutelage, and will be forever indebted to him for his kindness, compassion, and understanding.

I would also like to thank Stephanie Munger for her teaching and patience. Her technical expertise and help throughout the course of my dissertation research was invaluable. She helped me develop a more open perspective on life and learning.

I am also thankful for all the encouragement, guidance, and support that Jan Burt has provided me over the years. Without her, I likely would not have ventured into the wonderful field of connexin research. Many thanks as well to all the members of the Burt lab, both past and present. Their friendship helped keep me balanced and steady through the storm.

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Additionally, I'd like to thank the other members of my dissertation committee – Scott Boitano and Marlys Witte. They have been very generous with their time and guidance. They provided expertise that has served to increase the quality of my work.

I am also extremely grateful to the Physiological Sciences program as a whole. It has been a privilege to undertake my studies with such amazing faculty and staff.

Finally, I would like to thank my lovely lady, Liliana, for putting up with me for so many years while I worked towards finishing my dissertation. There were trying times, and while there were points in our relationship that strained and cracked, she was willing and patient enough to work together and mend them.

# DEDICATION

I dedicate this to my mother and father.

Mom, I never could have made it this far in life without your love and guidance.

In memory of my dad. You were taken away from us too soon. I wish you could've shared the joy of life with us.

## DESCRIPTION OF DISSERTATION FORMAT

This dissertation is based on the work that I carried out with my co-authors in three papers, which are attached at the end of this document as appendices. In chapters 2 through 4, I summarize the results presented in those papers and discuss their impact in the broader context of the fields of connexins and lymphatic developmental research. In chapter 5, I present concluding remarks and future directions for the investigation of connexins in lymphatic vascular development and function.

As to not duplicate figures within the text, I refer to the relevant figures in the aforementioned papers by placing a Roman numeral following the figure number to denote the paper in which they appear. For example, (Figure 2A; II) corresponds to Figure 2A of paper II. Roman numerals and the papers to which they reference are listed below:

I. **Kanady, J.D.**, Dellinger, M.T., Munger, S.J., Witte, M.H., Simon, A.M., 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.

II. **Kanady, J.D.**, Munger, S.J., Witte, M.H., Simon, A.M., 2014. Combining Foxc2 and Connexin37 deletion in mice leads to severe defects in lymphatic vascular growth and remodeling. Submitted to Dev. Biol. for review.

III. **Kanady, J.D.**, Simon, A.M., 2011. Lymphatic communication: connexin junction, what's your function? Lymphology 44, 95-102.

The following publication of which I am an author is referenced in the text, but the research done for that project does not constitute part of my dissertation thesis.

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

## STATEMENT OF CONTRIBUTIONS

For the studies presented in "Appendix I", I contributed to the collection and analysis of data pertaining to connexin expression in the murine embryonic lymphatic vasculature. I performed the majority of the quantitative analysis and three-dimensional reconstruction for the data regarding the jugular lymph sac. I also performed a portion of the Evans blue dye injections in the adult mice of that study, but the majority was performed by my co-authors. Quantitative analysis of the lymphatic valves in the mesentery and thoracic duct was performed wholly by my co-authors. Necropsy and documentation of chylothorax in the animals examined was performed mainly by my co-authors. I performed a majority of the experiments and analyzed all of the data for the study presented in "Appendix II". I also performed a majority of the writing, editing, and literature review for the article presented in "Appendix III". Unless otherwise specified, the use of "we", "us", and "our" in this dissertation refers to me and my co-authors on the above studies.

# Table of Contents

# ABBREVIATIONS

| | |
|---|---|
| **Ang1** | Angiopoietin - 1 |
| **Ang2** | Angiopoietin - 2 |
| **BEC** | Blood endothelial cell |
| **CD34** | Cluster of differentiation 34 |
| **Cx26** | Connexin 26 |
| **Cx37** | Connexin 37 |
| **Cx40** | Connexin 40 |
| **Cx43** | Connexin 43 |
| **Cx45** | Connexin 45 |
| **Cx47** | Connexin 47 |
| **ERK1** | Extracellular signal-regulated kinase 1 |
| **ERK2** | Extracellular signal-regulated kinase 2 |
| **Foxc2** | Forkhead box protein C2 |
| **GJ** | Gap junction |
| **IHC** | Immunohistochemistry |
| **JLS** | Jugular lymph sac |
| **LEC** | Lymphatic endothelial cell |
| **Lyve1** | Lymphatic vessel endothelial hyaluronan receptor 1 |
| **Nfatc1** | Nuclear factor of activated T-cells c1 |
| **Pecam1** | Platelet endothelial cell adhesion molecule 1 |
| **pHH3** | Phospho-Histone H3 |
| **Prox1** | Prospero homeobox 1 |
| **TEM** | Transmission electron microscopy |
| **VE-cadherin** | Vascular endothelial cadherin |
| **Vegfr1** | Vascular endothelial growth factor receptor 1 |
| **Vegfr2** | Vascular endothelial growth factor receptor 2 |
| **Vegfr3** | Vascular endothelial growth factor receptor 3 |
| **vWF** | von Willebrand factor |
| **Vegfa** | Vascular endothelial growth factor A |
| **Vegfc** | Vascular endothelial growth factor C |
| **Vegfd** | Vascular endothelial growth factor D |
| **PKB** | Protein kinase B |
| **PKC** | Protein kinase C |

# ABSTRACT

To maintain human health, the lymphatic system requires a structurally and functionally sound network of lymph vessels to absorb lipid-based nutrients, preserve extracellular fluid homeostasis, and mediate immune responses. Aside from lymphedema, investigations in the past few decades have found that impairment of the lymphatic vasculature is also involved in processes such as inflammation, tumor metastasis, fat metabolism, and obesity. However, despite a long history of study and rekindled vigor in the field of lymphatic vascular research, our knowledge of lymph vessel development and physiology is still quite limited. Recently, mutations in a protein family known as connexins (Cxs) were identified as the cause of lymphatic dysfunction in some cases of inherited lymphedema. This dissertation explores the role of primarily two specific connexins, Cx37 and Cx43, and the transcription factor Foxc2 in the morphogenesis and function of the lymphatic vasculature in mice. To accomplish this, phenotypic characterization of mice with genetic deficiencies (knockout mice) in Cx37, Cx43, and/or Foxc2 was performed principally via necropsy, histological techniques (immuno-fluorescence microscopy and H&E staining), and Evans blue dye (EBD) injections. Developmental abnormalities were found in lymphatic vascular growth, patterning, and remodeling in mice lacking Cx37, Cx43, Foxc2 or a combined deficiency of these proteins. Reductions or complete loss of lymphatic valves were a common finding in mice lacking one or more of these proteins. These valve deficits underlay lymphatic insufficiencies that resulted in lymphedema and chylothorax in some genotypes. Foxc2 was found to be a regulator of Cx37 expression. Moreover, Foxc2 was also dependent on Cx37 function for proper morphogenesis of lymph vessels. These findings pertaining to the expression of connexins in the lymphatic vasculature, their role in lymphatic valvulogenesis, and the interdependence of Cx37 and Foxc2 during lymph-vascular development represent my original contributions to human knowledge.

# CHAPTER 1: INTRODUCTION AND BACKGROUND

## The lymphatic system

### Discovery and early studies (circa 400 BCE to the 19th century)

The lymphatic system has been perhaps the most elusive system of the body, evading detection for millennia despite the powerful engine that underlies our desire to explore and discover – curiosity. The lymphatic system is mysterious, a storied biological system of the body that has a powerful allure in its peculiar anatomy and fascinating physiology. Unlike other systems of the body (of which their existence has been considered known since antiquity), the lymphatic system was *discovered*. Indeed, the attribution of the discovery of the lymphatic system and its various component structures has been the center of many disputes among scientists over the ages. Moreover, the origin of its development and functional importance are topics of controversy in the present day. This clash of intellects and egos seems to be a recurring theme in the history of science, and has perhaps served as some of the fuel that has propelled the advancement of our knowledge regarding the lymphatic system. Nevertheless, while many of the macro-level components of the lymphatic system have been described, knowledge of the micro and molecular-level aspects of lymphatic anatomy and physiology are still incomplete.

A number of authors credit Hippocrates (c. 460-370 BCE), the great Greek physician, with the initial discovery of the lymphatic system because of reference in his written works to "white blood" in the glands (Chikly, 1997; Choi et al., 2012; Grotte, 1979). However, other authors contend this point, and suggest that Hippocrates was instead referring to pus within infected lymph glands in the more superficial areas of the body (cervical, axillary, or inguinal regions) as opposed to the content within deeper mesenteric glands in the abdomen (Browse et al., 2003). Later, Aristotle (382-322 BCE) described "fibers containing colourless fluid between the blood vessels and the nerves" (Bartels, 1910). Though, even in this instance, there is disagreement regarding whether Aristotle was truly referring to the lymphatic vessels, due to uncertainty in the translation and precise meaning of the Greek word *ines* as "fiber" in Aristotle's writings (Kanter,

1987). While much of the written works by the ancient Alexandrian scholars have been lost to the ravages of war and time, the writings of the famed Greek physician, Galen (129 – c. 200 CE), contain quotations that indicate that the Alexandrians knew of the existence of the lymphatics within the mesentery. Galen referenced the teachings of the prominent Alexandrian physicians Herophilus (c. 335-280 BCE) and Erasistratus (c. 310-250 BCE), and their observations concerning the mesentery and intestine were certainly consistent with the description of lymphatic vessels. Following the time of Galen, the collapse of the Western Roman Empire around 500 CE marked the beginning of Europe's Middle Ages, which lasted until the 15th century. No evidence of advances in knowledge regarding the lymphatic system during that time have been found (Browse et al., 2003). This is perhaps unsurprising, as medical and scientific progress were stifled by the tumultuous sociopolitical climate during that time in Europe's history. Thus, the lymphatic system had been partially detected by the scientific and medical minds of the ancient world, but its function remained unknown.

The Renaissance (from the 14-17th century) marked a pivotal turning point in the freedom of thought and dissemination of ideas, during which time the lymphatic system was rediscovered. In 1622, Gasparo Aselli, an Italian anatomist and Professor of Anatomy and Surgery in Milan and Pavia, performed a vivisection of a dog to show the recurrent nerves to some friends. After viewing the nerves, Aselli opened the abdomen to watch the movements of the diaphragm. As he pulled down the intestine and stomach, he noticed numerous white cords throughout the mesentery. At first, he thought they were nerves, but when he found the actual nerves he realized that the white cords he saw were something wholly different. He then recalled arguments among anatomists about the function of the mesenteric vessels and decided to investigate further. Proceeding with the vivisection, he took a scalpel and cut into one of the cords. Once he saw the milky, white liquid gushing out, he knew he had seen something significant. He described the discovery in his book *De lactibus sive lacteis venis, quarto vascorum mesarai corum genere nova invento*:

> When I gathered my wits together for the sake of the experiment, having laid hold of a very sharp scalpel, I pricked one of those cords and indeed one of the largest

of them. I had hardly touched it when I saw a white liquid like milk or cream forthwith gush out. Seeing this I could hardly restrain my delight, and turning to those who were standing by, to Alexander Tadinus and more particular to Senator Septalius, who was both a member of the great College of the Order of Physicians and while I am writing this, the Medical Officer of Health, 'Eureka' I exclaimed with Archimedes, and at the same time invited them to see the interesting spectacle of such an unusual phenomenon. And they indeed were much struck with the novelty of the thing. (Aselli, 1628)

Aselli performed another vivisection the next day, but could not find the white vessels he had seen before. It occurred to him that the feeding/fasting state of the dog might be associated with the ability to see the vessels, and he performed yet another vivisection to confirm his hypothesis. Aselli went on to demonstrate this phenomenon in other mammals (cats, sheep, lamb, goats, cow, and horses). The drawings (Figure 1.1) based on this work that were made of the chylous lymphatic vessels of the mesentery are believed to be the first color plates in medical history (Browse et al., 2003; Kanter, 1987; Rusznyák et al., 2013). Aselli's findings were remarkable in that they were the first indication that one of the functions of the lymphatic system was linked to the processing of substances from the intestine and their passage to other regions of the body.

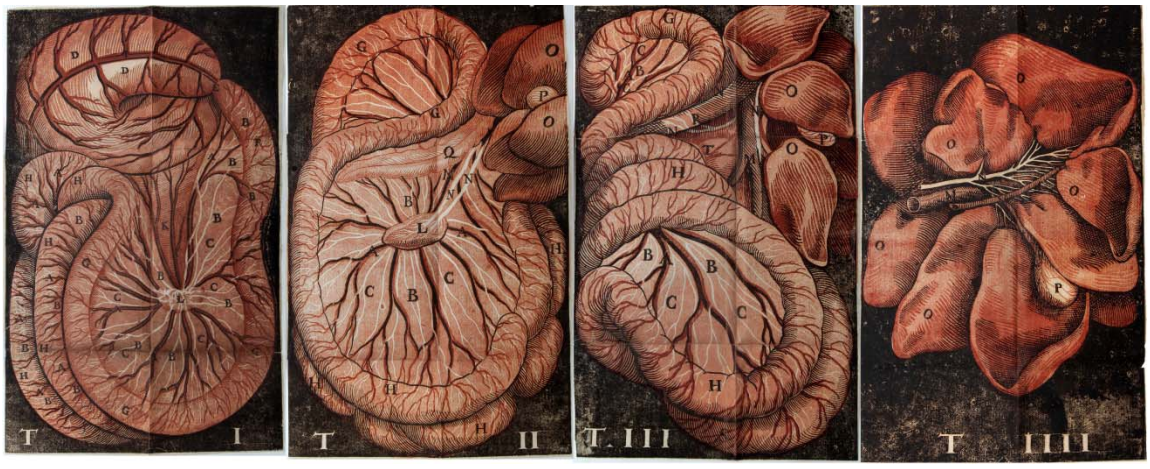


**Figure 1.1 –** Illustrations of the lymphatics of the intestines, mesentery (T. I, II, III), and liver (T. IIII) from Aselli's *De lactibus sive lacteis venis, quarto vascorum mesarai corum genere nova invento*. Lymphatic vessels appear in white, blood vessels in red. Aselli was unaware of the cisterna chyli and its drainage to the thoracic duct; he believed chyle went directly to the liver. Public domain resource, photographs courtesy of Christian Westergaard, Sophia Rare Books.

Through to the 16th and early 17th century, medical knowledge was still principally based on the teachings of Galen. One of the misconceptions passed on from Galen's teaching was that the liver was the site of blood synthesis in the body. With this idea in mind, Aselli had thought that the chyle from the intestines was passed through the lymphatic vessels to the liver, where it would be used to make blood. In 1628, shortly after Aselli's demonstration of the lymphatic vessels of the mesentery and intestine, William Harvey published his findings regarding the circulation of blood in his book *Exercitatio anatomica de motu cordis et sanguine in animalibus* (Harvey, 1628). Harvey's work and establishment of the idea of a circulatory system in the body was one of the earliest in a line of scientific discoveries that advanced a revolution of medical thinking. In 1651, Jean Pecquet, a French scientist, performed anatomical studies in the dog (and later in humans) describing the thoracic duct. He also showed that the chyle from the intestine and mesentery flowed through the mesenteric lymphatic vessels, to the cisterna chyli, tracing it through the thoracic duct to the site where it merged with the veins of the neck (Pecquet, 1651). With these observations, Pecquet linked the lymphatic vessels to the blood vessels. By extending his observations to the discovery of the blood circulation by Harvey, Pecquet described a system whereby nutrient absorption in the intestine passed to the lymph vessels and on to the blood for the nourishment of the body. Interestingly, Harvey was unimpressed by the discoveries of Aselli and Pecquet, and thought them to be unimportant for the treatment of diseases and of little relevance to the blood circulation (Chauvois, 1957). Unfortunately, this dismissive mindset and general disinterest within the scientific community regarding the lymphatic vasculature would continue over the next several centuries.

In 1652, Olaf Rudbeck, Professor of Anatomy in Uppsala, was invited to show to Queen Christina of Sweden a new set of vessels that he had discovered. In front of the royal court, he demonstrated the thoracic duct, lacteals, and peripheral lymphatic vessels – which at the time he called the vasa serosa. While Rudbeck was not aware of Pecquet's work regarding the thoracic duct, his findings regarding the peripheral lymphatic vessels were novel and noteworthy. Shortly after his demonstration to the royal court, Rudbeck would become embroiled in a lengthy disagreement with Thomas Bartholinus, Professor

of Anatomy in Copenhagen, regarding who should be credited with the discovery of the peripheral lymphatic vessels. In 1652, one month following Rudbeck's demonstration to Queen Christina, Thomas Bartholinus published his observations regarding the thoracic duct. Though, it was not until 1653 that Bartholinus published his work regarding the peripheral lymphatic vessels in a book entitled *Vasa lymphatic nuper Hafniae in animalibus inventa, et hepatis exsequiace*. Both men accused each other of plagiarism, fighting over credit for the find. Rudbeck, however, is recognized as the first to show the peripheral lymphatics, as a public demonstration to the royal court was the modern day equivalent to a journal publication. Regardless, it is interesting to note that Bartholinus is credited for the modern nomenclature of "lymphatic" vessels (Browse et al., 2003; Chikly, 1997; Kanter, 1987).

Curiously, despite the many advances in the identification of lymphatic vessels during the first half of the 17th century, lymphatic valves had not yet been clearly demonstrated. It was assumed that the lymphatic vessels contained valves based on analogies drawn from the blood circulatory system, of which it was known that the heart and veins contained valves. While some of the drawings from Rudbeck illustrated the lymphatic vessels with a beaded appearance, there was no bona fide demonstration of the existence of valves within the lymph vessels. One of the major complicating issues that contributed to this deficit in evidence at the time was the problem of tissue decay. There was a lack of effective preservation methods, and thus specimens could not be kept for long. Dissections needed to be performed each and every time the anatomy was to be taught or demonstrated. The precision of anatomical illustrations also suffered because of this limitation, since sketches of tissue architecture needed to be performed quickly by the anatomist (or via the commission of an artist) before the tissues degraded. Some anatomists circumvented this problem by performing vivisections of animals, but these studies were limited to those who could endure the suffering of the animal during the procedure (Kooijmans and Webb, 2011).[1]

[1] To highlight this point regarding vivisections, the reader should consider that effective general anaesthetics and procedures were not discovered and widely used until the early to mid 19th century (Dumas, 1932).

In the middle of the 17th century, a talented Dutch anatomist, Frederick Ruysch, employed a successful method of preservation and produced specimens that clearly demonstrated the multitude of valves that existed within the lymphatic vessels (Figure 1.2). Ruysch was driven by a long standing rivalry with another contemporary anatomist, Louis de Bils, who held the theory that no valves existed within the lymphatic vessels. Ruysch had developed a technique whereby he could ligate the lymphatic vessels, and using a very small blowpipe, inflate them with air. After this, he resected the lymph vessel and dried it, preserving it for later study. He had prepared several lymph vessels in this way, which contained some two thousand valves, to show de Bils and prove his theory incorrect. In 1665, Ruysch published his findings in a short work entitled *Dilucidatio valvularum vasis lymphaticis et lacteis*, providing the first clear illustrations of the lymphatic valves (Kooijmans and Webb, 2011; Ruysch, 1665).

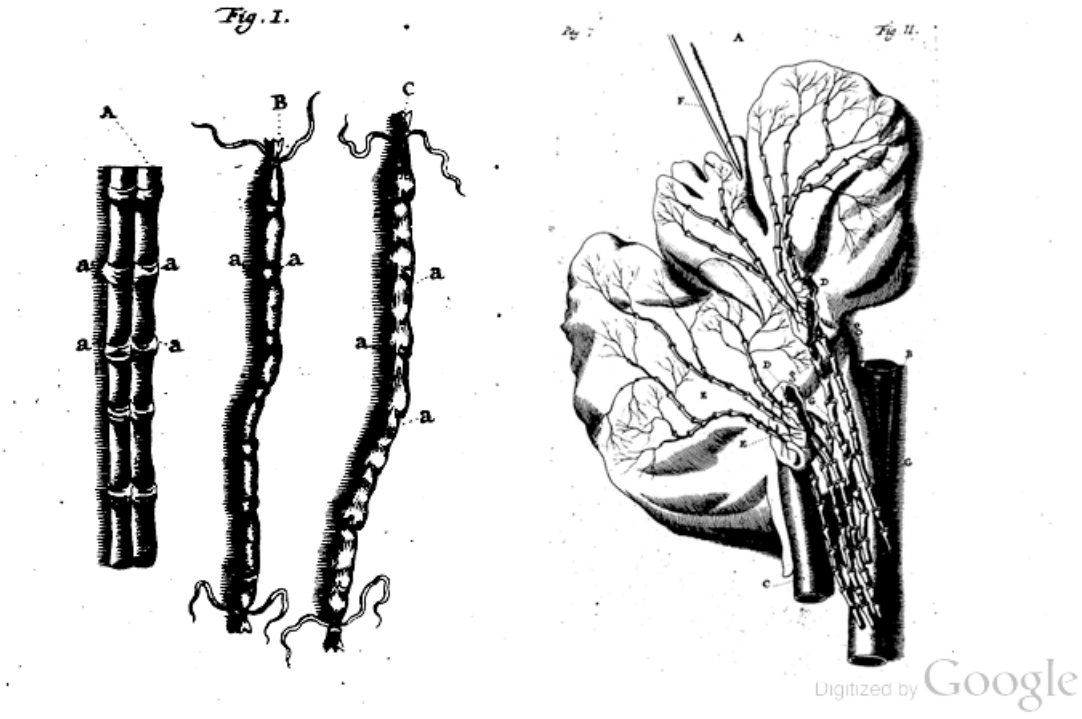


**Figure 1.2 –** Illustrations from Ruysch's book, *Dilucidatio valvularum vasis lymphaticis et lacteis*. *Left*: Ruysch prepared lymph vessels for preservation by ligating each end of the vessel and injecting them with air. *Right*: Valves within the hepatic lymph vessels of a horse. Public domain resource, however images were digitized by Google and thus retain the watermark

While anatomical studies were making significant headway in the identification of lymphatic vessels and their principal components, the function of the lymph vessels had remained difficult to ascertain. In 1654, Francis Glisson, a British physician, published a

book entitled *Anatomia hepatis*. In it, he proposed that the function of the lymph vessels was absorptive in nature, rather than simply being a continuation of the small arteries as was one of the ideas at the time. Roughly a century later, William Hunter, a Scottish anatomist described the function of the lymphatic system in returning fluid to the blood in a series of lectures beginning in 1746 (Browse et al., 2003). Two of Hunter's students, William Hewson and William Cruikshank, continued his work on the lymphatic system. They performed thorough anatomical studies by injecting mercury and other substances into tissues and observing their absorption into the lymphatic vessels in different areas of the body. Also, based on clinical observations, these investigators reasoned that the lymphatic system played a role in edema and the immune response. In 1786, the culmination of their findings were published by Cruikshank in one of the earliest comprehensive lymphatic anatomy and physiology texts, *The anatomy of the absorbing vessels of the human body* (Cruikshank, 1786; Kanter, 1987). Based on much of his work and that of his colleagues and predecessors, Cruikshank presented the primary functions which are ascribed to the lymphatic system today – absorption of tissue fluids and return to the blood, lipid-based nutrient absorption from the intestine, and defense against pathogens.

Work throughout the 17$^{th}$ to 19$^{th}$ centuries continued to augment knowledge regarding the anatomy of the lymphatic vasculature throughout the body. Researchers were injecting air, milk, dyes, wax, and mercury amalgams into the lymphatics to generate detailed anatomical maps (Browse et al., 2003). In particular, mercury injection (a technique pioneered in 1692 by Anton Nuck, a Dutch anatomist) was highly effective at visualizing the lymphatics and served as the primary form of lymphangiography in the following centuries (Nuck, 1692; Rusznyák et al., 2013). Honing this technique, Paolo Mascagni, an Italian physician, conducted studies in humans and published his seminal work on the anatomy of the lymphatic system in 1787, *Vasorum lymphaticorum corporis humani historic et ichnographia*, illustrated by Giro Santi. It remains one of the most detailed anatomical atlases of the lymphatic system to date, as impressive in the meticulous documentation of the lymph vessels and nodes as in the sheer artistic beauty of the illustrations (Figure 1.3). Through his extensive survey of the lymphatic vasculature, Mascagni also concluded that all lymph vessels must pass through at least

one lymph node in its course towards the venous system (Mascagni, 1787; Olry and Motomiya, 1997). This principle is considered "Mascagni's rule", though exceptions have been described (Rusznyák et al., 2013).

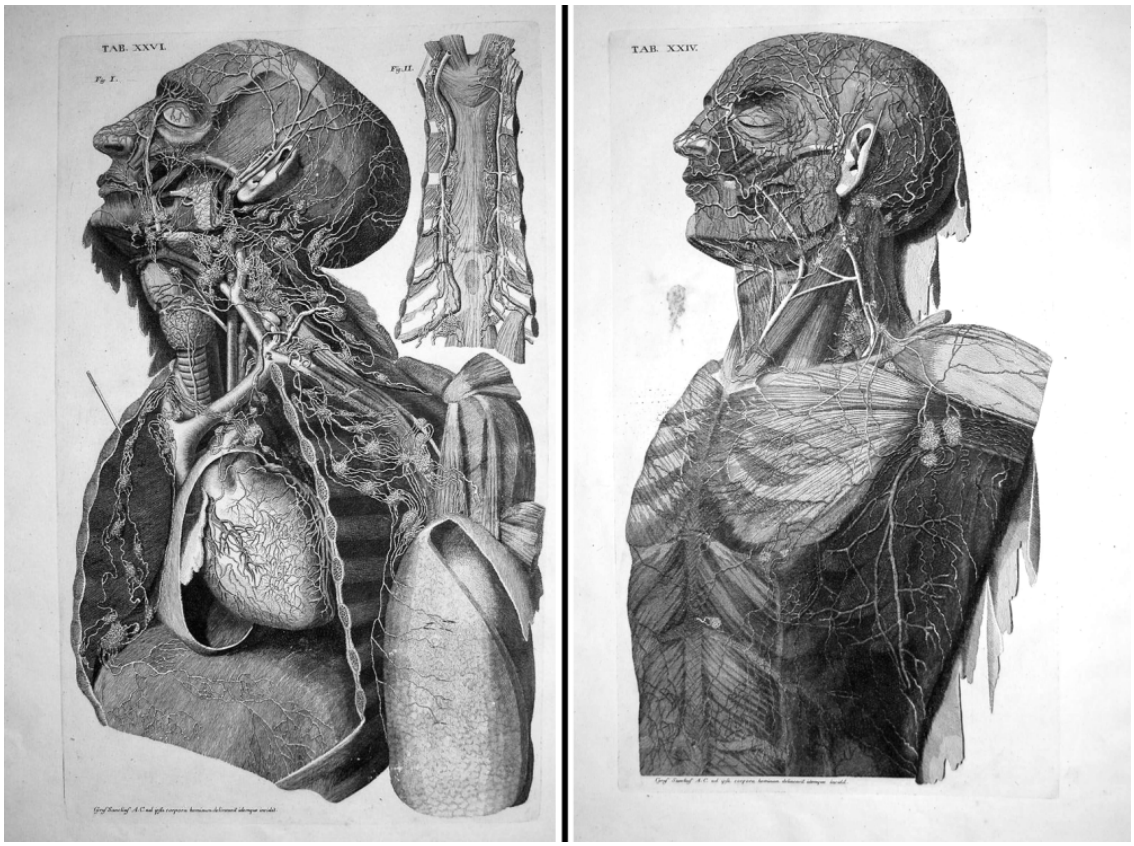


**Figure 1.3 –** Illustrations from Mascagni's text on lymphatic anatomy, *Vasorum lymphaticorum corporis humani historic et ichnographia*. Mascagni used mercury injections in order to visualize the lymphatic vessels (white vessels in the illustration). Images used courtesy of the University of Iowa John Martin Rare Book Room.

Marie Philibert Constant Sappey, a French anatomist, published a number of studies in the mid and late 19th century containing exquisite depictions of lymphatic anatomy (Figure 1.4), which were particularly exceptional for the quantification of lymphatic valves. Through his investigations in humans, he counted 60 to 80 valves spanning the lymph trunk of the upper limb and 80 to 100 valves in the trunk of the lower limb (approximately one valve per centimeter). Sappey also expanded the characterization of lymphatic anatomy in a number of tissues, and is considered one of the first to present clear descriptions of the superficial and deep lymphatics of the lung (Rusznyák et al., 2013; Sappey, 1874; Trapnell, 1965).

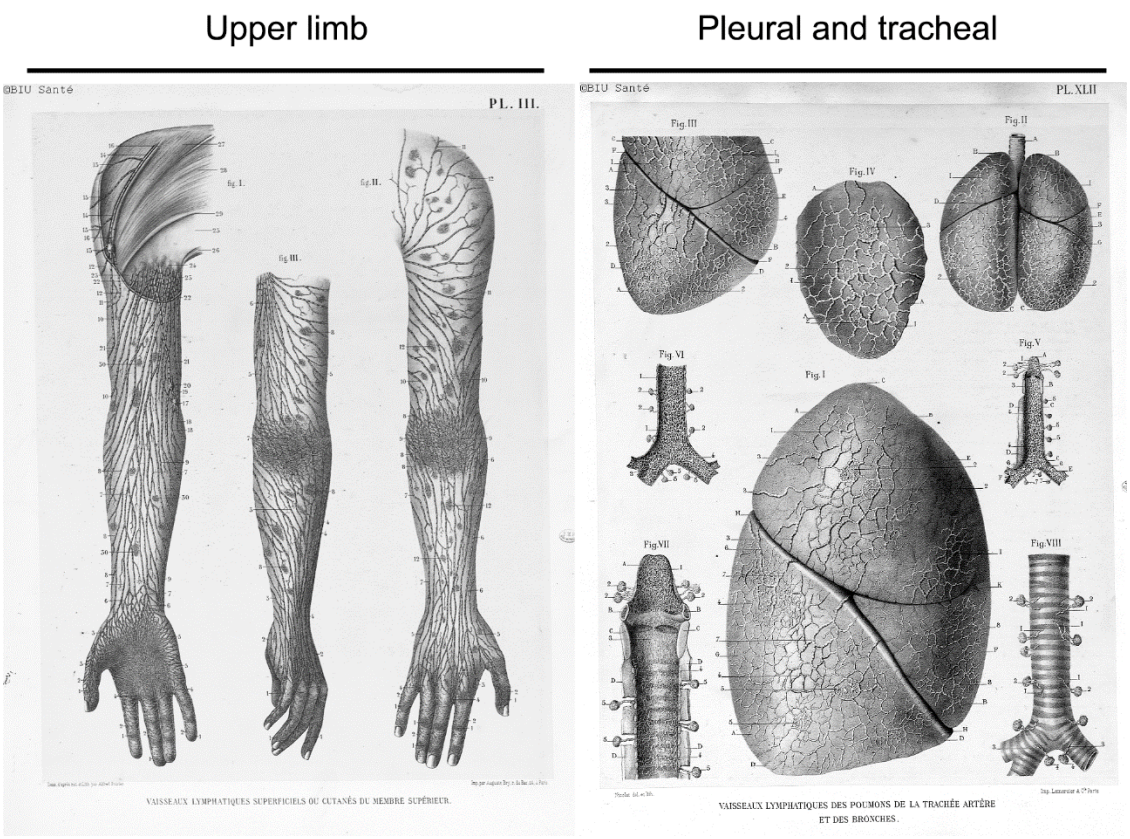


**Figure 1.4 –** Illustrations from Sappey's text on lymphatic anatomy, *Anatomie, physiologie, pathologie des vaisseaux lymphatiques considérés chez l'homme et les vertébrés*. Like Mascagni, Sappey used mercury injections to visualize the lymphatic vessels. Lymphatics of the upper limb and respiratory system are shown. Images from the digital archives of the Bibliothèque Interuniversitaire de Santé, Paris (BIU Santé), provided under open license by Etalab.

## Discoveries related to the physiology, microanatomy, imaging, and molecular identification of lymphatics (19th and 20th centuries)

The 19th and 20th centuries saw exciting advances in the understanding of the microanatomy and physiology of the lymphatic system. Some early ideas about how lymph ***moved*** through the lymphatic vasculature were provided by comparative anatomical investigations done by the German physiologist, Johannes Müller in 1833. He performed studies of the lymph hearts in amphibians, and showed that these "pulsating organs" were responsible for directing lymph through the system. By extension, it was hypothesized that a similar mechanism might exist in humans to propel lymph (Müller, 1833). In 1869, Arnold Heller observed rhythmic contractions of the lymph vessels in the guinea pig mesentery, and noted that the frequency of these

contractions was independent of the frequency of arterial pulsations and respiration (Heller, 1869). Heller's study is believed to be the earliest experimentally documenting the concept of the lymph pump in mammals and describing the intervalve segments as the functional unit of the pump (Aukland, 2005). Howard Florey, in 1927, confirmed Heller's experiments (Florey, 1927a), but also established that the rhythmic contractions were independent of the external nerves and suggested that the "stretch caused by filling and distension is essential for initiating contraction" (Florey, 1927b). Investigators in the following years reported similar contractions in lymph vessels in different species as well as in different regions of the lymphatic vasculature (Kinmonth and Taylor, 1956; Smith, 1949; Webb and Nicoll, 1944). In 1961, Mislin introduced a technique for isolation, cannulation, and perfusion of lymph vessels. Employing these techniques, he characterized the contraction pattern of the lymphatics under differing temperatures and feeding pressure, described action potentials of the lymphatics, and explored contractile responses in the presence of various drugs and hormones (Aukland, 2005; Mislin, 1961a, 1961b). Mislin also neologized the term "lymphangion" to describe the discrete segments between valves in the lymph vessel (Aukland, 2005; Mislin, 1961a). Later, it was demonstrated that spontaneous contractions of lymph vessel segments were initiated by a single propagated action potential (Allen et al., 1983), and these action potentials were the result of calcium-dependent, spontaneous transient depolarizations of myogenic origin (Van Helden, 1993). Substantial advances in the understanding of contraction propagation, coordination between lymphangions, and lymphatic pacemaker activity were made near the end of the 20$^{th}$ century (McGeown et al., 1987a, 1987b; McHale and Meharg, 1992; McHale and Thornbury, 1989; Zawieja et al., 1993).

While the aspects of lymph propulsion were starting to be elucidated in the 19$^{th}$ century, the actual mechanism by which lymph was ***formed*** remained an enigma. Heller's contemporary colleague, Carl Ludwig, proposed that intracapillary blood pressure forced fluid through the wall of the blood vessel to produce lymph – an extension of his thesis work that was the foundation of the concept of glomerular filtration, known as the "filtration hypothesis" (Browse et al., 2003; Davis et al., 1996; Ludwig, 1858). However, another German physiologist, Rudolf Heidenhain, conducted a set of experiments in which the results could not be explained by the filtration hypothesis

alone. He had found that when the inferior vena cava was obstructed, there were proteins in the thoracic duct lymph that were in higher concentration than their simultaneous concentration in the blood plasma. Thus, in 1891, he proposed that there was an active process (secretion) carried out by the capillary in order to concentrate those substances in the interstitium (and consequently the lymph), forming the basis of the "secretory hypothesis" of lymph formation (Heidenhain, 1891; Henderson, 2013). This idea of secretion from the capillary was later demonstrated to be incorrect when Ernest Starling showed that the high concentration of protein in thoracic duct lymph could be prevented by the simultaneous occlusion of the lymphatics from the liver and the inferior vena cava (Starling, 1894). Therefore, most of the protein in the thoracic duct lymph was coming from the liver and could reasonably be explained by a difference in the permeability of liver capillaries to protein. Starling would later establish that proteins (blood plasma and interstitial) were critical to determining fluid movement across the capillary wall, a principle that forms the foundation of our current understanding of blood capillary dynamics, lymph formation, and extracellular fluid balance (Starling, 1909, 1896).

In addition to lymph propulsion and formation, the route by which lymph and larger particles ***entered*** the lymphatic vessels from the interstitium was an issue that was being investigated. In 1933, Drinker and Field proposed the following:

> A further possibility, for which no proof exists, is that the delicate lymph capillaries are fixed to surrounding tissues by fine strands of reticulum, and that muscular movements by pulling on these strands may induce distortion and temporary openings, through which fluid enters eventually to reach a valved trunk from which escape does not occur. (Drinker and Field, 1933)

Morphological evidence to support the above idea came in 1935, when Pullinger and Florey performed histological studies on the lymphatic vessels of the mouse ear showing that fibers of the surrounding connective tissue were attached to the lymphatic capillary endothelium (Pullinger and Florey, 1935). However, advances beyond light microscopy were needed to effectively pursue the issue. Just four years earlier in 1931, Ruska and Knoll constructed the first transmission electron microscope, an invention that would

increase the resolving power of microscopy by several orders of magnitude (Bogner et al., 2007). Further development of electron optics and the eventual commercial availability of electron microscopes allowed the detailed study of lymphatic endothelial cells (LECs) and their organization relative to the extracellular matrix. In 1961, Casley-Smith and Florey investigated the ultrastructure of lymphatic capillaries, and the electron micrographs they published showed gaps between LECs as well as "bundles of collagen fibres invested" in them (Casley-Smith and Florey, 1961). Later studies by Leak and Burke continued exploring the ultrastructure of the lymphatic endothelium, and clearly demonstrated filaments that anchored the cells to the surrounding connective tissue (Leak and Burke, 1968, 1966). Studies by Hogan and Unthank, and later Aarli and Aukland, provided experimental data linking anchoring filaments, increased interstitial volume, and filling of initial lymph vessels (Aarli and Aukland, 1991; Hogan and Unthank, 1986; Unthank and Hogan, 1988).

The 20$^{th}$ century also brought about many important advances in the clinical assessment of lymphatic vascular function. In 1927, Moniz developed x-ray angiography, a revolutionary new technique that allowed the visualization of the cerebral blood vasculature (Ligon, 1998). However, this method did not work for the lymphatic vasculature (Browse et al., 2003). Early anatomical studies used colored dyes to highlight the lymph vessels, but these substances only allowed short-lived glimpses of the lymphatics since they would quickly diffuse from the vessels. In 1933, Hudack and McMaster experimented on themselves by performing intradermal injection of Patent Blue Violet to visualize the lymphatics (Hudack and McMaster, 1933). This technique was later used as a preliminary step by Kinmonth to identify lymph vessels prior to the injection of radioactive contrast material, thus establishing a new protocol for lymphangiography and the assessment of lymphatic vascular dysfunction in human patients (Kinmonth, 1952).

In addition to new imaging modalities such as electron microscopy and lymphangiography, the development of labeled antibodies by Coons in the 1940s and the use of immunohistochemistry (IHC) heralded a new era of molecular biology. IHC introduced the ability to exquisitely discriminate between cells and tissue types based on their unique expression of biological molecules (most typically proteins) (Matos et al.,

2010). Discovered in the 1970s, two of the earliest markers that were used to identify endothelial cells were angiotensin converting enzyme (ACE) and von Willebrand factor (vWF), a blood glycoprotein (Bloom et al., 1973; Caldwell et al., 1976; Hoyer et al., 1973; Risau, 1995). Other endothelial cell markers such as vascular endothelial growth factor receptors 1 (Vegfr1) and 2 (Vegfr2), vascular endothelial cadherin (VE-cadherin), platelet-endothelial cell adhesion molecule-1 (Pecam1; also known as CD31), P-selectin, and CD34 were found and characterized in the decades following (Bonfanti et al., 1989; Breier et al., 1995; Fina et al., 1990; Hsu-Lin et al., 1984; Lampugnani et al., 1992; Newman et al., 1990; Peters et al., 1993; Vecchi et al., 1994). While some of these endothelial cell markers are expressed by both blood endothelial cells (BECs) and LECs (VE-cadherin and Pecam1) (Dejana et al., 2009), the expression pattern is usually not sufficient to reliably distinguish between BECs versus LECs. Consequently, the detection of these markers was usually taken as positive identification of BECs.

The discovery of markers specific to the lymphatic vasculature lagged behind for nearly two decades. In 1995, one of the first lymphatic markers, vascular endothelial growth factor receptor 3 (Vegfr3) was identified (Kaipainen et al., 1995). However, it soon became clear that Vegfr3 was important in not only the lymphatic vasculature but also the blood vasculature, as mice lacking Vegfr3 died during embryonic development due to cardiovascular defects (Dumont et al., 1998). Nevertheless, Vegfr3 becomes largely restricted to the lymphatics in adult tissues, and as such is an effective lymphatic marker. In 1999, the lymphatic endothelial hyaluronan receptor (Lyve1) was identified in LECs (Banerji et al., 1999), but was also later found in macrophages (Schledzewski et al., 2006) and liver sinusoidal endothelium (Mouta Carreira et al., 2001). Podoplanin, a cell surface glycoprotein, was another of the earliest discovered lymphatic markers, and from characterizations thus far seems to be restricted to the lymph rather than the blood vasculature (Breiteneder-Geleff et al., 1999; Wetterwald et al., 1996). Also near the very end of the 20$^{th}$ century, the homeobox transcription factor, Prox1, was found to be prominently expressed by LECs (Wigle and Oliver, 1999). Among endothelial cells, Prox1 was presumed to be specific to LECs (Wigle and Oliver, 1999), but was later found to be expressed by the endothelial cells of the venous valves as well (Bazigou et al., 2011). In fact, depending on the level of the vasculature and the embryonic/adult

time point examined, many of the endothelial markers that have been found to date have overlapping expression in both BECs and LECs (Sleeman et al., 2001). Therefore, it has often been necessary to use two different molecular markers in order to positively identify LECs from BECs. The expression patterns and utility of these markers in distinguishing lymphatic versus blood vessel continues to be an active area of investigation.

Our knowledge of the lymphatic system has grown tremendously over the course of human history. Its anatomy has been explored, carefully documented, and beautifully illustrated by many talented researchers. Basic observations regarding its physiology formed the foundation from which the understanding of its function has expanded. Exciting technological advancements have aided our ability to visualize the lymphatics, though classic techniques are still effective and enjoy frequent use in the scientific literature. Work during the last two decades has revealed numerous proteins that have been useful for the microscopic differentiation of lymphatic vessels from blood vessels, and have also enabled targeted genetic experimentation in cell and animal models. Importantly, determining the precise functions of the proteins that comprise the unique molecular complement of the lymphatic system will be essential to elucidating the biological mechanisms that contribute to its structure and function (in both normal physiologic and pathophysiologic settings). While certain aspects of the anatomy and physiology of the lymphatic system are still incomplete, much of the research in the past ten years has been focused on the processes that direct the development and growth of the lymphatic vasculature. Of particular interest has been the issue of tumor lymphangiogenesis and its role in the dissemination of cancerous cells, but developmental and pathological lymphangiogenesis are also of high clinical relevance.

*The following sections in this chapter will address the anatomy and physiology of the lymphatic system as we currently understand it, discuss fundamental concepts of lymphatic development, and introduce the molecular focus of my research – connexin proteins and the Foxc2 transcription factor.*

## Functional overview of the lymphatic system

The lymphatic system is composed of a vasculature and specialized lymphatic organs. The lymphatic vasculature is a vast network of vessels throughout the body, linking a collection of some 600-700 lymph nodes, and connects to the blood vasculature in a number of limited, discrete locations (namely, near the subclavian and jugular veins). The lymphatic organs consist of the red bone marrow, thymus, spleen, lymph nodes, and mucosa-associated lymphatic tissues (such as the tonsils in the oral mucosa, Peyer's patches in the intestinal mucosa, and bronchus-associated lymphatic tissue in the respiratory mucosa). Together, this group of vessels and organs carry out a number of functions critical to sustaining life: mediating immunity and resistance to disease, transporting lipid-based nutrients absorbed from the intestines, and maintaining fluid homeostasis in the body (Földi, 2006).

### *Immune function*

The lymphatic system is the immune system. The lymphatic organs listed above comprise the body's defense system, protecting it from both exogenous (i.e. pathogens, foreign substances) and endogenous (cancerous cells) threats. The major cellular component of the lymphatic system that carries out this defense function is the lymphocyte. Lymphocytes are divided into two broad classes, T- and B-lymphocytes, which are involved in cell-mediated and antibody-mediated immune responses respectively. Approximately 500 billion lymphocytes are housed throughout the human body, only 2% of which circulate through the blood at any given time. The other 98% reside within the lymphatic organs and traffic between tissues via the lymphatic vessels (Trepel, 1974).

### *Dietary fat transport*

The lymph vessels of the intestine are responsible for transport of lipid soluble nutrients that have been absorbed from the intestine. Lipids (cholesterol, long chain fatty acids) and lipid-soluble vitamins (vitamins A, D, E, and K) that are absorbed by

enterocytes (epithelial cells lining the intestinal mucosa) are packaged into structures called chylomicrons. From the enterocyte, chylomicrons pass to the interstitial space within the submucosa and subsequently enter the lacteals. Lacteals are the initial lymphatics of the small intestine, which traverse the center of each intestinal villus. Chyle, the lipid rich lymph that forms in the lacteals, moves through a network of lymph vessels within the intestinal wall, continuing through the mesentery and the chain of lymph nodes within it, eventually reaching the major lymphatic trunks and ultimately emptying into the blood. Through this pathway, lipids from the diet are transported to the blood and provide nourishment for the cells of the body. From a pharmacological perspective, the lymphatic system also represents an important route for the absorption of orally administered lipophilic drugs and water-insoluble peptide-like molecules (Yáñez et al., 2011).

### *Extracellular fluid homeostasis*

Large, multi-cellular organisms such as humans require a cardiovascular system to efficiently deliver substances (nutrients, gases, ions, hormones, etc.) to different areas of the body. The cardiovascular system consists of the heart and an extensive network of blood vessels serving nearly every tissue in the body. As blood carries the aforementioned substances to the tissues, they can diffuse (along with water) across the capillary endothelium and into the interstitium in order to bring nourishment to the tissues. To accomplish this, the heart must pump and exert a physical driving force to move blood through the system. This pumping action produces a blood pressure which also generates a force directed against the walls of the vessels which contain it. In effect, the blood pressure within the capillary can "push" water and dissolved substances out of the vessel and into the interstitial space (a process termed ultrafiltration).

Proteins also play a pivotal role in the delivery of substances to the tissues. Acting as "vehicles" (Westphal, 1971), plasma proteins carry hormones, vitamins, metals, and fatty acids with them as they transit out of the blood vessel via diffusion or ultrafiltration. The blood capillaries have a varying degree of permeability to proteins depending on the specific capillary bed (Asscher and Jones, 1965). The capillaries within the brain have the lowest permeability, while those within the liver (sinusoids) have the

highest. Importantly, proteins exert an osmotic force (colloid osmotic pressure within the blood plasma and interstitium) which also influences water movement. Proteins within the blood will act to keep water within the vessel, whereas proteins in the interstitium will act to draw water out of the vessel (Starling, 1896). Therefore, the formation of interstitial fluid is determined by the balance of forces working to direct water into the blood vessel and those working to direct it out. Overall, based on the magnitude of these forces along the length of the capillary, there is a net movement of water and proteins out of the blood and into the interstitium.

The lymphatic vasculature functions to prevent the buildup of protein and fluid in the interstitium, and is thus critical to maintaining extracellular fluid homeostasis. If the lymph vessels do not return extravasated proteins to the blood and fail to avert the accumulation of proteins within the interstitium, the protein concentration in the interstitium would approach that of the blood plasma. The difference in colloid osmotic pressure between the interstitial fluid and the blood would then collapse, and fluid reabsorption into the blood capillaries would cease. This would lead to edema (tissue swelling). Additionally, if the lymph vessels were also unable to return sufficient protein and fluid back to the blood circulation, blood volume would plummet and hypovolemic shock would result (Földi, 2006).

## Architecture of the lymphatic vasculature

Lymphatic vessels can be broadly grouped into absorbing vessels and conducting vessels. **Lymphatic capillaries** and **precollectors** comprise the absorptive side of the system whereas **precollectors** and **collectors** constitute the conducting side of the system. Lymphatic capillaries drain lymph into precollectors which lead into a network of collectors running parallel to the capillary plexus. Lymph passes through a series of lymph nodes, entering them through afferent lymph vessels and exiting through efferent lymph vessels. The confluence of efferent lymph vessels forms the larger lymphatic trunks as lymph proceeds through the lymphatic vasculature. The trunks drain into two major ducts – the thoracic duct and right lymphatic duct – which join with the venous blood supply in the region of the venous angle.

### *Lymph*

Lymph is largely considered to be identical in composition to interstitial fluid, though there is some indication it can be modified by LECs or fluid extravasation from the lymph vessel. It is typically a proteinaceous, clear or straw-colored fluid. However, the composition of lymph differs depending on the region of the body. For example, lymph from the liver is higher in protein content while lipid-rich lymph (chyle) from the intestines varies significantly in composition depending on the feeding state of the animal (Földi, 2006).

### *Lymphatic capillaries*

The initial lymph vessels are the lymphatic capillaries. Lymphatic capillaries can be found in all vascularized tissues with the exception of the central nervous system and bone marrow. These blind-ended vessels are composed of an endothelium and an incomplete basement membrane (Casley-Smith and Florey, 1961). The endothelium of the lymphatic capillary is attached to the surrounding connective tissue via anchoring filaments (Leak and Burke, 1968, 1966). These anchoring filaments allow the lymphatic capillary to respond to changes in interstitial fluid volume and promote lymph formation (Hogan and Unthank, 1986). Under non-filling conditions, LECs are arranged such that they overlap each other like shingles (His, 1863). However, increases in interstitial fluid volume result in mechanical stretching of the anchoring filaments, which in turn pull on their insertion points on LECs to reveal interendothelial gaps.

Interestingly, cell-cell junctions between LECs of lymphatic capillaries are discontinuous, and they are arranged such that adherens junctions and tight junctions are concentrated in focal points resembling buttons. Regions between these button-like junctions form membrane flaps that act as valve-like structures (Baluk et al., 2007). The opening of these inlet valves facilitates the movement of water and larger particles (such as cells and proteins) from the interstitium and into the lymphatic vessel during its filling phase.

*Lymphatic precollectors*

The bridge between lymphatic capillaries and collectors are the precollectors. Accordingly, these vessels have characteristics between that of capillaries and collectors. They are similar to lymphatic capillaries in that some portions of the precollector have a discontinuous basement membrane and possess anchoring filaments; however, other portions may have smooth muscle coverage and contain intraluminal valves. Because of these characteristics, precollectors may function in either an absorbing or conducting capacity. In terms of cell-cell junctions, it is also intriguing to note (compared to other lymph vessel types) that some authors have found that contacts between endothelium and smooth muscle are extremely frequent in precollectors (Sacchi et al., 1997).

*Lymphatic collectors*

The major fluid conducting components of the lymphatic vasculature are the collectors. These vessels have smooth muscle coverage and abundant intraluminal valves. Valves are critical in preventing backflow, and ensure that net movement of lymph proceeds toward the junction between the lymphatic and blood vasculature. In humans, valves are encountered approximately every centimeter along the superficial collectors of the upper and lower limbs (Sappey, 1874). The region between two valves is termed a "lymphangion", and this structure forms the contractile subunit of the collecting vessel (Mislin, 1961a). Smooth muscle coverage is concentrated around the length of vessel between valves, with a paucity of smooth muscle coverage in the region of the valve sinus. The lymphangion allows for the active propulsion of lymph, generating the pressure needed to move lymph from the periphery to the venous blood centrally.

Morphologically, when examined in cross section, collectors are similar in appearance to veins and contain three histologically identifiable tunics (an intima, media, and externa). The basement membrane is continuous and multiple layers of smooth muscle cells surround the vessel. Zipper-like junctions (similar to those between BECs) are present between LECs of collectors. Occludin, claudin-5, and VE-cadherin are found in these junctional areas (Baluk et al., 2007). The organization of these cell-cell junctions

limits fluid egress from the vessel and corresponds well with the fluid conducting role of collecting lymphatics.

### *Lymphatic valves*

A distinctive characteristic of the lymphatic vasculature is the presence of numerous intraluminal valves in the conducting vessels. The most common type of intraluminal lymphatic valve is the bicuspid valve (Figure 1.5 D-E), with unicuspid and tricuspid valves occurring more infrequently (Kampmeier, 1928). Additionally, based on three-dimensional reconstructions of lymphatic valves from both light and electron microscopy, Daróczy describes four main types of intraluminal valves: joining valves, segment valves, unicellular valves, and bunch valves. Joining valves occur at the confluence of two separate lymphatic vessels. Segment valves are found within the length of a single vessel, and subdivide the vessel into lymphangions (as mentioned above). Unicellular valves are single endothelial cells that protrude into the lumen of the vessel. Bunch valves have a connective tissue core attached to the perivascular interstitium, while the free edge of the valve consists of endothelial cells (tip cells) that fan out into the lumen and resemble flowers in a bunch (Daróczy, 1988).

Structurally, the typical lymphatic valve is composed of a folded layer of endothelial cells that extends into the lumen. Thus, in relation to flow, the valve endothelium is divided into an upstream and a downstream side. The endothelium lining each side of the valve leaflet is mechanically supported by an intervening connective tissue core that is continuous with the perivascular interstitium. Valve LECs are attached to the underlying extracellular matrix (ECM) core via integrins (e.g. containing α-5, α-6, and α-9 subunits). The core itself consists of a number of different ECM proteins including collagen IV, fibronectin (and the EIIIA splice isoform), tenascin-C, and laminin containing the α5 subunit (Bazigou et al., 2009; Norrmen et al., 2009).

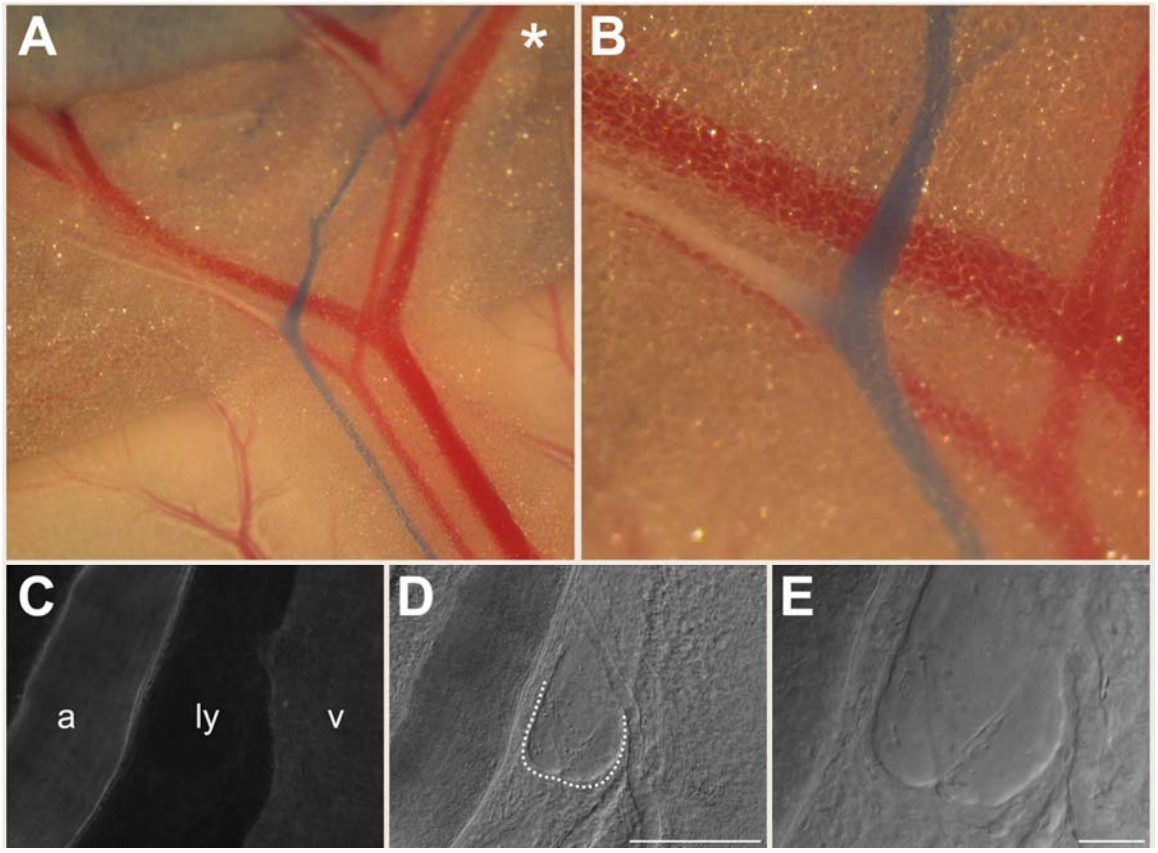


**Figure 1.5 –** Lymphatic valves are uniquely structured to prevent backflow and ensure unidirectional flow. Blood vessels (red) and lymph vessels (white) of the mesentery are shown in (A) and (B). 1.5-2 hours after feeding with corn oil, the abdomen of an anaesthetized mouse was opened to view the mesenteric lymphatic vessels (which appeared milky white due to being filled with chyle). In this example, Evans blue dye (EBD) was injected in the submucosa of the intestine and its clearance from the intestinal parenchyma by the lymphatics was traced. Dye flowed from the area of injection (asterisk, A) towards the bottom part of the field. A competent valve at the branch point helped maintain one-way flow and prevented dye reflux back towards the intestine, as seen in (A) and at higher magnification in (B). A fresh, un-fixed preparation from the mesentery was mounted on a slide and viewed with a fluorescence microscope. Arteries (a) and veins (v) were autofluorescent (due to elastic fibers within their walls), which facilitated their identification (C). A lymphatic (ly) vessel was situated between the two blood vessels. Differential interference contrast (DIC) optics allowed for the visualization of the valve sinuses and leaflet (D, highlighted with the dotted line). Higher magnification of the valve region is shown in (E). Note, direction of lymph flow in C-E is from the bottom of the image to the top. Scale bars: (D) 200 μm, (E) 50 μm.

*The combination of the above vascular elements from lymphatic capillary to collector creates a hierarchical network that complements the blood vasculature. In fact, the blood vasculature relies on a competent lymphatic vasculature to recycle extravasated proteins and maintain blood volume, traffic cells of the immune system between tissues, and deliver lipid-based nutrients to the cells of the body. The principal cells of the lymph vessel (LECs, smooth muscle, and pericytes) are physically linked to each other via cell-cell and cell-matrix adhesion molecules in a structurally precise manner that reflects*

*distinct functional specialization in every level of the lymphatic vasculature. The coordination of these cell-cell and cell-matrix interactions are critical to ensuring proper vessel function, and are also crucial for the correct developmental sequences that lead to lymphatic vascular formation.*

## Lymphatic vascular development

Two different views on the development of the lymphatic vasculature have been debated for more than a century (Witte et al., 2001). One view, the centrifugal theory, proposes that the entire lymphatic vasculature has a venous origin – lymph sacs arise from the veins and sprouts from these structures create the lymph vessels that spread into the tissues of the body (Sabin, 1909, 1904, 1902). The second view, the centripetal theory, contends that lymph vessels form independently of the veins and are derived from the mesenchyme (similar in principle to blood vasculogenesis, de novo formation of blood vessels) (Kampmeier, 1912). In essence, the difference between these ideas can be condensed to whether the lymphatic vasculature initially develops centrally and grows towards the periphery (centrifugal) or whether lymph vessels are formed in the periphery and grow towards the central veins (centripetal).

Much of the disagreement between the proponents of each theory stem from the experimental techniques used to support their conclusions. Data were collected either via dye injections or through serial sectioning and reconstructions of tissue architecture from embryonic samples. Historically, injections of dye (or classically mercury) were the primary means of illustrating the anatomy of the lymphatic system. Therefore, among some anatomists, evidence of lymphatic vessels was taken only if they could be shown to be injected. However, the counterargument to this position was that injections could only be made into a continuous system of vessels. Hence, the inability to visualize lymphatics via injection was not evidence of their absence and isolated, immature lymphatics in the form of mesodermal clefts could still exist. There have been over a hundred publications since the turn of the 20$^{th}$ century regarding the topic, and the issue is still not completely resolved (Rusznyák et al., 2013). Nevertheless, examining the introductory remarks regarding lymphatic development in a number of recent articles reveals that a venous

origin to the lymphatics is the prevailing view adopted by many investigators. While genetic lineage tracing studies have provided firm evidence for the venous descent of the lymphatic vasculature (Srinivasan et al., 2007), whether the ***entire*** mammalian lymphatic system is ***solely*** derived from venous endothelial cells remains a point of contention. Indeed, other sources of LECs during postnatal lymphatic development have been found such as macrophages (Maruyama et al., 2005) and bone marrow cells (Kerjaschki et al., 2006; Religa et al., 2005; Salven et al., 2003). Furthermore, mesenchymal cells with lymphoendothelial characteristics have been characterized and are believed to integrate with the lymph sacs during murine embryonic development (Buttler et al., 2006). Also, the use of mesenchymal stem cells for lymphatic regeneration has been explored (Conrad et al., 2009), demonstrating an interest in mesenchymal contributions to postnatal lymphatic vessel growth.

### *Lymphvasculogenesis*

The process of *de novo* formation of lymphatic vessels is termed **lymph-vasculogenesis**, and it is directed by a coterie of proteins that orchestrates a series of precisely controlled events during embryogenesis. Three transcription factors have been shown to be essential for early LEC differentiation from venous BECs – Sox18, COUP-TFII, and Prox1. Mice in which these proteins have been ablated fail to develop a lymphatic vasculature. DNA binding studies have demonstrated that Sox18 and COUP-TFII directly regulate the homeobox transcription factor Prox1 (a key regulator of LEC identity) (François et al., 2008; Srinivasan et al., 2010; Wigle and Oliver, 1999). Prox1 was found to be expressed in a subset of endothelial cells within the cardinal veins starting around embryonic day (E) 9.5, which are subsequently induced to sprout from the vein by vascular endothelial growth factor C (Vegfc) and form the primitive lymph sacs (Karkkainen et al., 2004; Wigle et al., 2002). In a recent study, Prox1 has been found to directly regulate Vegfr3 (the receptor for which Vegfc is a primary ligand) in LEC progenitors. Moreover, the same study demonstrated that Vegfc-mediated activation of Vegfr3 signaling forms a feedback loop that is responsible for maintaining Prox1 expression in LEC progenitors and consequently lymphatic identity (Srinivasan et al., 2014).

*Lymphangiogenesis*

Whereas lymphvasculogenesis leads to the incipient generation of lymph sacs, **lymphangiogenesis** results in the expansion and restructuring of those sacs into a mature vascular network. This process is achieved through sprouting, branching, and remodeling of pre-existing lymphatic vessels in response to signals from peripheral tissues and organs. Despite a recent surge of interest in lymphangiogenesis, much of our understanding of vessel growth stems from a longer history of the study of blood angiogenesis. However, while similar molecules and morphological changes are involved in both blood angiogenesis and lymphangiogenesis, the two processes are distinct and differentially regulated on both spatial and temporal levels.

In terms of cellular signaling pathways, two of the key receptors involved in lymphangiogenesis are Vegfr2 and Vegfr3 as well as their ligands, Vegfc and Vegfd (Achen et al., 1998; Joukov et al., 1996). The signaling elicited by these receptors leads to PKC (protein kinase C) mediated activation of ERK1/ERK2 (extracellular signal-regulated kinases 1 and 2) and phosphorylation of PKB (protein kinase B) (Mäkinen et al., 2001; Shibuya, 2013). Additionally, Vegfr2 can be activated by Vegfa which regulates proliferation and migration of LECs through ERK1/ERK2 and PI3-K (phosphoinositide 3-kinase) mediated phosphorylation of PKB (Dellinger and Brekken, 2011). Neuropilin 2 (Nrp2), a co-receptor for Vegfr3, can also modulate LEC responses to Vegfc and Vegfd (Kärpänen et al., 2006; Xu et al., 2010). Other growth factors such as Ang1 (Angiopoietin-1), Ang2 (Angiopoietin-2), FGF (fibroblast growth factor), HGF (hepatocyte growth factor), IGF-1 (insulin-like growth factor 1), IGF-2 (insulin-like growth factor 2), and PDGF-BB (platelet-derived growth factor BB) stimulate lymphangiogenesis, though the precise mechanisms by which they direct lymph vessel growth are still being investigated (Björndahl et al., 2005; Cao et al., 2004; Kajiya et al., 2005; Morisada et al., 2005; Tammela et al., 2005).

Sprouting and branching of new vessels during lymphangiogenesis involves tip cell to stalk cell differentiation. This process is similar to the events that occur during blood angiogenesis, where Vegfa serves as a guidance cue for the extension of filopodia from tip cells on the advancing edges of the vascular network. The stalk cells, on the other hand, increase their proliferative activity in response to growth signals to increase

the size of the network (Gerhardt et al., 2003). Work done with mouse retinal vasculature has determined that the appropriate number of tip cells to stalk cells is regulated through Notch dependent signaling (Hellström et al., 2007; Suchting et al., 2007). Though studies investigating this process in LECs are limited, it has been found that tip to stalk cell differentiation is under the control of Vegfr3 and involves modulation of Notch signaling (Tammela et al., 2011; Zheng et al., 2011).

During lymphangiogensis, another crucial series of molecular events ensures that the lymphatic vasculature remains separated from the blood vasculature, except in a number of distinct locations. These so called lymphovenous junctions are primarily found in the vicinity of the venous angles, where the thoracic and right lymphatic ducts drain into the venous blood. Interestingly, growing evidence reveals that platelets are the prominent player in this process of lymph-blood separation (Hess et al., 2014). Molecules such as podoplanin, platelet C-type lectin-like receptor 2 (CLEC2), spleen tyrosine kinase (SYK), and SH2 domain containing leukocyte protein of 76kDa (SLP-76) are involved maintaining lymph-blood separation, as evidenced by the occurrence of blood-filled lymphatics in mice with genetic deficiencies in the aforementioned proteins (Bertozzi et al., 2010; Finney et al., 2012; Fu et al., 2008; Suzuki-Inoue et al., 2010). Additionally, a potentially non-platelet mediated mechanism for keeping lymph and blood vessels distinct from each other during postnatal lymphatic development involves fasting-induced adipose factor (Fiaf) in the small intestine (Bäckhed et al., 2007).

### *Lymphatic vascular remodeling and valvulogenesis*

After the events of lymph-vasculogenesis and lymphangiogenesis initially establish a primary lymphatic plexus, remodeling must occur to create a phenotypically distinct hierarchy of vessels (capillaries, precollectors, and collectors). Early studies employing ink injections into the skin of fetal pigs revealed that a primary lymphatic plexus is first laid down at the interface between the dermis and hypodermis. Following this, lymph vessels sprout from the primary plexus and spread throughout the dermis to form the lymphatic capillaries. Concurrently, the primary plexus transforms to adopt a collecting vessel phenotype (Sabin, 1904). In the small intestine, a similar sequence occurs where a primary plexus forms within the submucosa, and sprouting from the

primary plexus generates the initial lymphatic vessels (which include the lacteals) within the mucosa. As is the case in the skin, the lymphatic network matures, and the primary plexus in the intestine remodels into a network of collecting vessels (Heuer, 1909). More recent investigations using genetically modified mice lend further support to this model of development. Mice lacking the PDZ domain of ephrin-B2 (a transmembrane ligand for the Eph receptor tyrosine kinase) develop a primary lymphatic plexus, but they fail to form a secondary plexus of initial lymphatics (Mäkinen et al., 2005). $Ang2^{-/-}$ mice have lymphatic vascular remodeling defects, which manifest as a dramatic reduction of lymphatic valves, formation of a dysplastic primary lymph plexus with premature recruitment of smooth muscle cells, and failure to properly develop a secondary plexus of initial lymphatics (Dellinger et al., 2008; Gale et al., 2002). Thus, separate molecular pathways govern the formation of the primary lymphatic plexus versus its remodeling and maturation into a functional network.

One of the hallmark features of lymphatic collecting vessels are valves, which are formed during remodeling of the primary lymphatic plexus. In a classic study examining serial histological sections of human embryos at different stages of development, Kampmeier proposed that three different modes of lymphatic valvulogenesis existed. The first method involves the growth of one lymphatic vessel into another, with the valve leaflets being formed by the protrusion of the invading vessel into the lumen of the other. The second method entails the formation of valve leaflets via the proliferation of LECs at the confluence of vessels at an existing vessel branch. In the third method, LECs in a ring-like orientation around the vessel proliferate and extend into the lumen to form the valve (Kampmeier, 1928). Kampmeier suggested that this "origin as endothelial thickenings" also pertained to venous valves as well (Kampmeier and La Fleur Birch, 1927). Regardless of the type of valve being formed, Kampmeier noted that the placement of valves and their genesis were likely governed by mechanical events such as movements of the organism during embryogenesis and fluid (lymph or blood) flow dynamics (Kampmeier and La Fleur Birch, 1927; Kampmeier, 1928).

Despite their paramount importance to lymphatic vascular function, interest in exploring the morphogenesis of valves has only recently been revitalized. Evidence from immunohistochemical evaluation of lymphatic valves supports Kampmeier's third

mechanism of valvulogenesis, and experiments done in cell culture with LECs extend the idea of mechanically-transduced signals and flow influencing lymphatic valve development (Sabine et al., 2012). Additionally, using immunohistochemistry and scanning electron microscopy, work by Bazigou and colleagues elegantly demonstrated a similar developmental sequence for venous valve development. Bazigou et al. also discovered that many of molecular markers of lymphatic valves (Prox1, integrin-α9, ephrin-B2, and Fibronectin-EIIIA) were also found in venous valves (Bazigou et al., 2011). Moving forward, the characterization of genetically engineered mice will continue to provide insight into mechanisms controlling valvulogenesis – for both lymphatic and venous valves. Indeed, deficits in valve development have been found in mice lacking integrin-α9, Foxc2, ephrin-B2, and Ang2 (Bazigou et al., 2009; Dellinger et al., 2008; Gale et al., 2002; Mäkinen et al., 2005; Petrova et al., 2004). Determining the contribution of these proteins will further our understanding of the mechanisms governing valvulogenesis and will hopefully lead to treatments for complications related to insufficient valve function such as lymphedema and varicose veins.

*Significant progress towards understanding the ontogenesis of the lymphatic vasculature has been made within the last few decades. This has been due in large part to the recent identification of molecular markers of lymph vessels. These biological molecules have facilitated the microscopic characterization of lymphatic vessels through immuno-histochemical techniques as well as enabled the generation of genetic mouse models to explore lymphatic development. Despite the surge of studies investigating lymphatic developmental processes and how they contribute to overall lymph-vascular function and human health, our knowledge is still woefully incomplete. We contribute to this growing body of knowledge by identifying two connexins, Connexin37 (Cx37) and Connexin43 (Cx43), as part of the ensemble cast controlling lymphatic development. We have found that these proteins are indispensable for normal lymphangiogenesis and lymphatic remodeling. The following section will introduce the basics of their molecular anatomy and present an overview of their cellular function.*

## Connexins

We live in the information age. As a result of advancements in digital technologies, namely computers and the internet, the speed of communication has dramatically increased. Overall, human society is more "connected" now than ever before. Our ability to communicate over long distances, rapidly respond to changing situations, and coordinate with one another has transformed our way of life. When we step back and look at the human race as a whole, we exist as a multi-organismic entity in certain respects – from the level of a neighborhood, city, nation, or world. This idea of a collective existence is made possible by robust interpersonal communication systems and underlies our ability to thrive. It is also largely responsible for the celerity with which we generate new knowledge and pass it on. Interestingly, when we consider life on the cellular level, an information revolution on the micro-scale was a requisite step in going from unicellular to multicellular organisms. Indeed, cell-cell communication is considered a requirement for multicellular organization (Grosberg and Strathmann, 2007). One of the biological innovations during evolution that facilitated this transition was the gap junction. In deuterostomes (a phylum of animals which includes all chordates, such as humans), gap junctions allow for direct cell-cell communication and are formed by a family of membrane proteins called connexins.

### Brief history of gap junctions and the discovery of connexins

Some of the earliest evidence for gap junctions came from observations of direct electrical coupling between excitable cells (cardiac cells and select neurons) (Furshpan and Potter, 1957; Weidmann, 1952). In the first half of the 20$^{th}$ century, one of the hotly debated topics in neuroscience concerned whether synaptic transmission was chemically or electrically based. Based on work done by Henry Dale and Otto Loewi identifying acetylcholine as a naturally occurring substance and its effect on the amphibian heart, the idea of chemical transmission at the synapse was firmly rooted (Tansey, 2006). However, in the 1950s, definitive evidence was found to support electrical transmission. Furshpan and Potter, recording electrical impulses from crayfish giant neurons, discovered that the delay between pre- and post-synaptic neurons involved in escape

responses was extremely short (~0.1 ms). This delay was about an order of magnitude shorter than reported delays at chemical synapses (~2 ms). Furshpan and Potter also determined that electrotonic conduction (passive spread of charge) occurred across the synapse, a phenomenon that was not explainable by chemical transmission mechanisms (Furshpan and Potter, 1959, 1957). For this to occur, electrical charge would need to pass directly from one neuron to another without the cell membranes as intervening insulators. Their findings provided solid evidence for the existence of electrical synapses. These electrical synapses were later shown to be formed by innexins (proteins analogous to connexins, but found in invertebrates) (Phelan et al., 1998).

Shortly after Furshpan and Potter's findings were published, studies by Bennett and colleagues at the end of the 1950s and through the 1960s done in pufferfish provided some of the earliest evidence of electrical synapses in vertebrate animals (Bennett et al., 1963, 1959, 1967). During this time, observations of cell-cell passage of large metabolites and dye transfer also provided evidence for intercellular coupling between nonexcitable cells (Loewenstein and Kanno, 1964; Subak-Sharpe et al., 1969). In the early 1960s, Dewey and Barr observed what they believed were membrane fusions (which they termed a nexus) in smooth muscle, cardiac muscle, and epithelia from various species (Dewey and Barr, 1964, 1962) that were similar to the membrane fusions observed in pufferfish by Bennett and colleagues (Bennett et al., 1963). These membrane areas, however, were indistinguishable from tight junctions in early ultrastructure studies. Revel and Karnovsky later developed a technique that allowed for the distinction to be made between tight junctions and these reported areas of membrane fusion. By using a membrane-impermeable colloidal form of lanthanum as a contrast agent to mark the extracellular space, the resulting electron micrographs revealed extremely small gaps between cells in the area of the nexus (Revel and Karnovsky, 1967). This characteristic was the basis for the naming of "gap" junctions.

Follow-up studies employing the recently invented freeze-fracture technique demonstrated exquisite polygonal lattices at gap junctional areas, providing evidence that led to the proposition that membrane-spanning proteins from adjacent cells formed channels and were the basis of this type of intercellular communication (Goodenough and Revel, 1970; Kreutziger, 1968a, 1968b). Later, methods for isolating gap junctions were

developed by exploiting their insolubility in non-ionic detergent (Goodenough and Stoeckenius, 1972). Using these methods, Goodenough isolated large amounts of pure gap junctions from liver, which allowed initial analyses to be conducted on their component proteins via SDS-PAGE (sodium dodecyl sulfate – polyacrylamide gel electrophoresis). In the publication of those results, Goodenough proposed the current name of vertebrate gap junction proteins – connexins (Goodenough, 1974). Further improvements in methods for protein isolation from gap junctions led to higher yields, which facilitated subsequent amino acid sequencing and the creation of antibodies (Singh and Malhotra, 1996). In the mid 1980s, Paul as well as Kumar and Gilula isolated the first cDNA sequences for connexins (Kumar and Gilula, 1986; Paul, 1986). Studies throughout the latter half of the 20$^{th}$ century and early 21$^{st}$ century used multiple techniques (x-ray diffraction, cryoelectron microscopy, x-ray crystallography, circular dichroism, substituted-cysteine accessibility method, and nuclear magnetic resonance) to further understand the secondary, tertiary, and quaternary structure of connexins and the channels they form (Beyer et al., 1987; Cascio et al., 1990; Kronengold et al., 2003; Kumar and Gilula, 1992; Makowski et al., 1977; Oshima et al., 2007; Perkins et al., 1997; Skerrett et al., 2002; Unger et al., 1999; Unwin and Zampighi, 1980; Yeager, 2009; Zampighi and Unwin, 1979; Zhou et al., 1997).

*While many advances have been made in determining the molecular structure of connexins, data is still lacking regarding the differences between specific connexin isoforms. Ongoing work continues to generate finer structural resolution of these proteins at the atomic scale. An overview of our current understanding of the molecular anatomy of connexins will be presented in the next section.*

## Nomenclature and molecular anatomy

Among vertebrates, there are over 20 connexin isoforms, each with different tissue expression profiles. The various members of the connexin protein family are named according to their predicted molecular mass in kDa. For example, Cx37 and Cx43 are approximately 37 and 43 kDa, respectively. There are differences in molecular mass between some species; however, clear orthologs are identifiable based on degree of

sequence identity (e.g. human Cx62 is orthologous to mouse Cx57). Despite species differences, there is a high level of sequence identity between human and mouse connexins such that many isoforms have similar molecular masses. For instance, human Cx37 and Cx43 are orthologous to mouse Cx37 and Cx43 (Beyer and Berthoud, 2009). Because of the similarities in sequence as well as tissue distribution between human and mouse connexins, the study of connexins in mouse models is considered highly relevant for understanding their role in human health and disease.

Connexins contain four membrane spanning domains (M1-M4) with cytoplasmic carboxyl terminal (CT, also referred to as cytoplasmic tail) and amino terminal (NT) ends. Two extracellular loops (E1-E2) and one cytoplasmic loop (CL) comprise the other structural regions of the protein (Figure 1.6A). The extracellular loops are the most conserved regions between connexins, with the transmembrane spanning domains and NT relatively conserved among connexins. The CT and CL are highly variable between connexins, and these differences are responsible for the broad range of molecular masses across various isoforms (Beyer and Berthoud, 2009). More importantly, dissimilarity of the CT and CL among connexin isoforms underlies differences in protein regulation and consequently function (Dbouk et al., 2009).

Connexins have diverse channel-forming capabilities and can adopt multiple different channel configurations due to the structural conservations mentioned above. Connexin-based channels are formed by the oligomerization of six individual connexin proteins into a structure termed a connexon. Connexons can be composed of a single connexin isoform (homomeric), but cross-compatibility of some connexins also allows them to assemble as a mix of isoforms (heteromeric). A connexon within the membrane of one cell can dock with a connexon from an adjacent cell to form what is called a gap junction channel. Gap junction channels can be composed of connexons of the same type (homotypic) or different types (heterotypic). Clusters of gap junction channels aggregate within the membrane to create plaques that bring cell membranes into extremely close apposition (within a few nanometers). Finally, undocked connexons within the membrane are called hemichannels (half of a gap junction channel) (Yeager, 2009). Figure 1.6B-C provides a graphical presentation of these concepts.

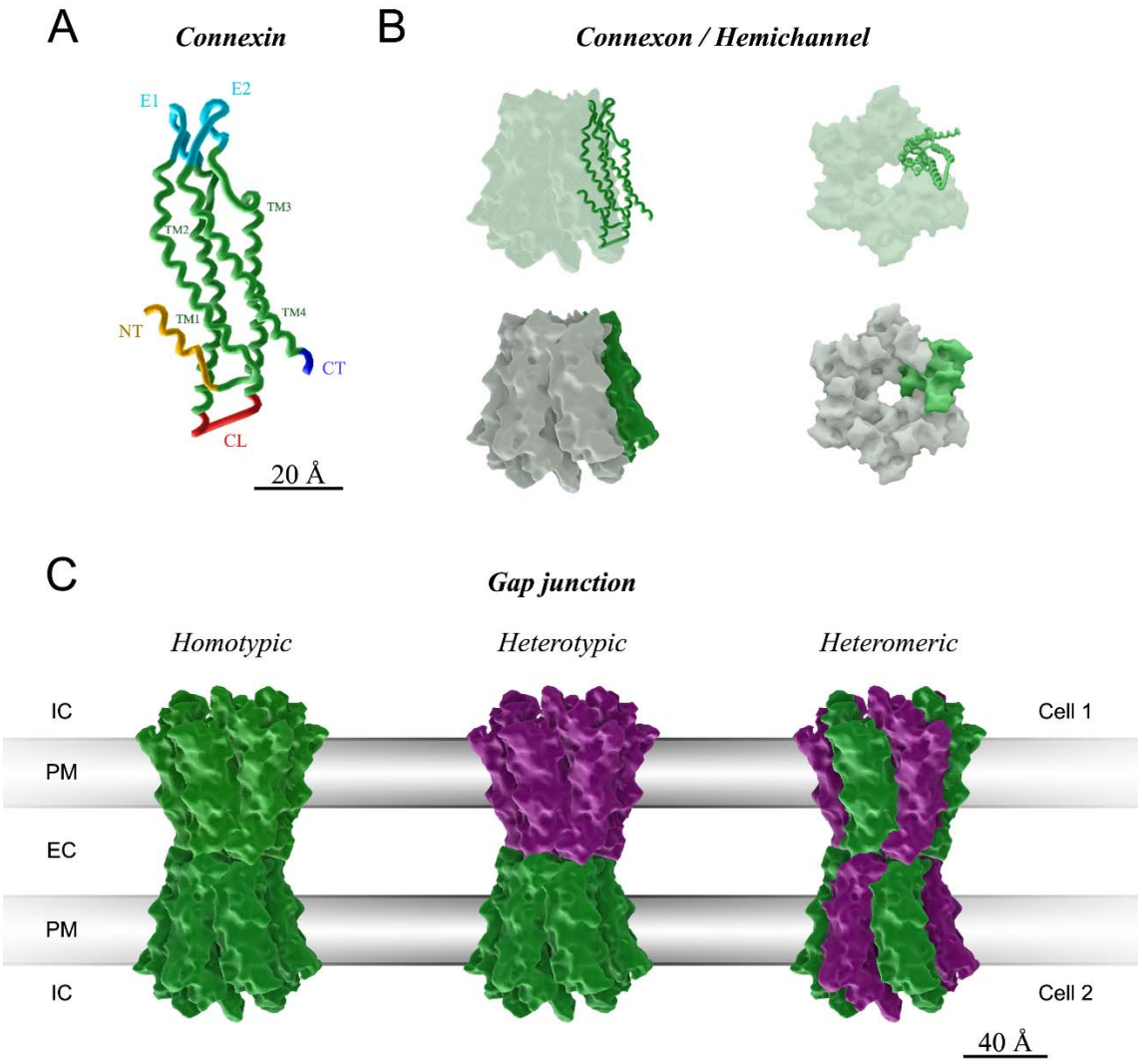


**Figure 1.6 –** Molecular anatomy of connexins, hemichannels, and gap junction channels. The connexin protein can be divided into the following components, as shown in ribbon form in panel (A): two extracellular loops (E1/E2, teal), four membrane-spanning domains (TM1-TM4, green), a carboxyl terminus (CT, blue), an amino terminus (NT, yellow), and a cytoplasmic loop (CL, red). Six connexin proteins assemble together to form a connexon (also known as a hemichannel). Shown in the top row of panel (B) are side (left) and top (right) views of a connexon, one connexin subunit is shown in ribbon form while the other subunits are shown as transparent surface representations. In the bottom row of (B), all subunits are displayed as surface representations, with the corresponding subunit from the top row in green. Note the six-fold symmetry of the connexon. Gap junction channels are formed by the docking of two connexons from adjacent cells. Shown in panel (C) are side views of a gap junction channel within the membrane of two neighboring cells (cell 1 and 2). A gap junction channel can be homotypic or heterotypic. Heteromeric connexons can also form gap junction channels. IC, intracellular; PM, plasma membrane; EC, extracellular. Scale bars: (A) 20 Å, (B-C) 40 Å. Models are based on the 3.5 Å resolution crystal structure of Cx26 (Maeda et al., 2009), PDB (Protein Data Bank) ID: 2ZW3.

*The molecular structure of connexin proteins affords them a high degree of flexibility in their assembly into channels. The large diversity in channel configurations likely contributes to the variety of functional processes in which connexins participate. In the*

*following chapters that discuss the original research I and my co-authors conducted, it will be valuable to keep in mind that both Cx37 and Cx43 have heteromeric and heterotypic channel compatibility with one another (Brink et al., 1997; Yeager and Nicholson, 2000). In the following section of this chapter, cellular functions of connexins will be presented, highlighting Cx37 and Cx43 functions as determined by gene knockout and cell culture studies.*

## Cellular functions

Connexins mediate cell-cell communication in vertebrate animals by forming intercellular channels (gap junction channels) that provide for continuity between the cytoplasmic compartments of adjacent cells. In general, gap junctions allow cells to coordinate their responses by facilitating rapid, synchronized activity as well as permitting cells to share signaling molecules and metabolites. A prime example of connexin function is in the coordinated beating of the heart, where these proteins allow the rapid conduction of electrical impulses critical to the synchronization of myocardial contraction. However, connexin-based intercellular communication is not limited to the passage of charge-carrying ions. Cellular metabolites, signaling molecules, short peptides, and short nucleotide sequences in the form of miRNA and siRNA can also pass between cells through these intercellular channels (Kizana et al., 2009; Neijssen et al., 2005; Valiunas et al., 2005). Although it is often noted in the literature that connexin-based channels have a size limit of approximately 1 to 1.5 kilodaltons (kDa) for permeants (Dbouk et al., 2009), the latter examples of miRNAs and siRNAs (which are much greater than 1.5 kDa) indicate that molecular shape may be another important factor to consider. Thus, in the case of coordinating activities in non-excitable cells, the specific function of gap junction channels depends on the identity of the permeant. Unfortunately, the identification of specific physiological gap junction permeants *in vivo* has been a challenging task, which has hampered the determination of the precise molecular pathways that connexins influence.

In addition to forming intercellular channels, connexins can also form hemichannels that allow substances (ATP, glutamate, prostaglandins) to pass from the cytosol to the extracellular space (Cotrina et al., 1998; Jiang and Cherian, 2003; Ye et al.,

2003). In these scenarios, hemichannels may be mediating intercellular signaling via a paracrine mechanism. There is increasing evidence for hemichannel activity in multiple cell types (cardiomyocytes, astrocytes, osteocytes, neurons) as well as a growing interest in their role in pathophysiological states (Contreras et al., 2002; DeVries and Schwartz, 1992; Hofer and Dermietzel, 1998; Kondo et al., 2000; Li et al., 2001).

Furthermore, a growing number of connexin-associated proteins are being identified via co-localization and co-immunoprecipitation studies, such as cytoskeletal elements (α- and β-tubulin, actin), tight junction components (zonula-occludens 1 [ZO-1] and 2 [ZO-2], occludin, claudin), adherens junction proteins (cadherins, catenins), serine/threonine kinases (PKC) and tyrosine kinases (c-Src, v-Src) as well as serine/threonine and tyrosine phosphatases (for a review, see Dbouk et al., 2009). Based on the wide variety of interacting proteins, channel-independent roles for connexins in regulating cellular function may be quite diverse. Indeed, both channel-dependent and channel-independent roles in cellular function have been identified in processes such as growth, tumorigenicity, apoptosis, migration, and differentiation (Hirschi et al., 2003; Olk et al., 2009; Squecco et al., 2006; Vinken et al., 2011; Zhou and Jiang, 2014).

### *Cx37 and Cx43*

Cx37 is expressed in varying degree by a number of different cell types from tissues and organs such as the ovary, bone, skin, lung, blood vessels, heart, and kidney (Goliger and Paul, 1994; Haefliger et al., 2000; Pacheco-Costa et al., 2014; Simon and McWhorter, 2002; Simon et al., 1997; Stoessel et al., 2010; Traub et al., 1998). In the reproductive system, Cx37 is critically important for the differentiation of ovarian follicles, as evidenced by infertility in female mice lacking Cx37. Cx37 has also been found to influence bone development, and mice with global deletion of Cx37 have higher bone mineral density (Pacheco-Costa et al., 2014). In the blood vasculature, combining genetic loss of Cx37 and Cx40 results in abnormal blood vascular development, and recent data from our lab show that Cx37 knockout mice fail to develop venous valves (Munger et al., 2013; Simon and McWhorter, 2002). Interestingly, loss of Cx37 also enhances vascular growth in mice after surgically-induced hindlimb ischemia (Fang et al., 2011). Furthermore, investigations done in cell culture with rat insulinoma cells have

found that Cx37 causes growth arrest, an effect that requires a fully functional channel (Burt et al., 2008; Good et al., 2014, 2011). Together, these studies implicate Cx37 in a wide range of cellular functions, such as growth, tumor suppression, differentiation, and possibly cell migration (based on its influence on vascular development).

Cx43 is the most ubiquitously expressed connexin and can be found in at least 35 different tissues and cell types in the body (Laird, 2006). Early studies of Cx43 demonstrated that it is critical for embryonic development, as mice with global deletion of Cx43 die perinatally due to obstruction of the pulmonary outflow tract (Reaume et al., 1995). In a later study, delayed ossification and skull malformations were also found in $Cx43^{-/-}$ mice (Lecanda et al., 2000). Because of the embryonic lethality of global knockout of Cx43, the generation of tissue-restricted knockout mice has been necessary to explore Cx43 function postnatally. Mice with cardiac-specific knockout of Cx43 have significant reduction in ventricular conduction velocity and die from spontaneous ventricular arrhythmias by two months of age (Gutstein et al., 2001). In mice with endothelial-specific deletion of Cx43, hypotension and bradycardia result (Liao et al., 2001). Cx43 is also involved in wound healing in the skin, where it is proposed to coordinate the proliferation and mobilization of keratinocytes (Kretz et al., 2003). Loss of Cx43 in astrocytes causes enhanced spreading depression (neuronal inactivation associated with epileptiform activity migraine) and locomotory activity (Theis et al., 2003). In mice with myometrium-specific ablation of Cx43, parturition is delayed, suggesting that Cx43 is critical to the regulation of uterine smooth muscle contractility (Döring et al., 2006). Similar to Cx37, Cx43 is also expressed in granulosa cells where it is required for folliculogenesis (Ackert et al., 2001; Juneja et al., 1999). Expression of Cx43 in HeLa cell lines has also been associated with diminished growth capacity (King et al., 2000). As a consequence of its wide tissue distribution, Cx43 is involved in numerous functions. From the studies above, some of the most prominent roles for Cx43 involve cardiovascular development, cardiac conduction, modulation of neuronal pathways, smooth muscle contractility, growth and migration related to epithelial repair, and follicular differentiation.

Cx37 and Cx43 are also involved in human health and disease. Through a number of epidemiological studies, a single nucleotide polymorphism in the gene coding

for Cx37 (C1019T) has been associated with atherosclerosis, carotid intima-media thickness, coronary artery disease, and myocardial infarction in various human populations (Meens et al., 2012). As for Cx43, over 70 mutations in humans have been linked to oculodentodigital syndrome (ODD; OMIM 164200), a disease affecting the morphology of the eyes, teeth, and fingers (Laird, 2014).

*Many of the known functions for specific connexins have been determined by examining the phenotype of disorders caused by connexin mutations, investigating the effect of their loss in animal models, and exploring their functional characteristics in cell culture. In chapter 3, I will present our findings regarding the loss of Cx37 and/or Cx43 on lymphatic vascular development and function in mice. In the next and last section of this chapter, I will introduce forkhead transcription factors. Emphasis will be placed on Foxc2, a specific forkhead transcription factor that regulates Cx37 expression in the lymphatic endothelium (Kanady et al., 2011; Sabine et al., 2012).*

## Forkhead transcription factors

Transcription factors are largely responsible for the unique identity of various cell types through their ability to regulate gene expression. They accomplish this by binding to DNA in response to certain stimuli (mechanically-induced signaling, for example), promoting the recruitment of RNA polymerase, leading to transcription and production of mRNA, and ultimately dictating the complement of cellular proteins (Latchman, 1997). Approximately 7% of all mouse genes code for transcription factors, resulting in a myriad of these proteins with diverse tissue expression profiles and functions (Gray et al., 2004). The importance of transcription factors to biological function has been demonstrated time and again in knockout models and overexpression systems. The specific transcription factor that my studies on lymphangiogenesis have focused on is forkhead box C2 (Foxc2).

Foxc2 is a member of the forkhead family of transcription factors. Forkhead transcription factors share a highly conserved DNA-binding domain, and are so named because of ectopic head structures that develop in the fore- and hindgut of fruit flies with

mutations in the first identified gene of this family (Weigel et al., 1989). Based on sequence homology, there are 19 subclasses of forkhead box proteins denoted A through S. To date, there have been over 40 forkhead box genes identified in mammals, which are expressed in nearly every cell and tissue of the body. A wealth of studies have determined that forkhead transcription factors have essential functions in cell-cycle regulation, cell survival, metabolism, immunoregulation, and embryonic development (Carlsson and Mahlapuu, 2002; Wijchers et al., 2006).

## Foxc2 – identification and function

Foxc2 was first discovered in the 1990s, where its expression was initially found in non-notochordal mesoderm as well as the mesoderm of the trunk, head, and limbs (Miura et al., 1993). Since then, Foxc2 expression has been identified in many nonaxial mesoderm and neural crest-derived tissues. In the developing heart, Foxc2 can be found in endothelial and mesenchymal cells, second heart field progenitors, and the proepicardium. In other areas of the cardiovascular system, Foxc2 is found in the endothelium of developing arteries and veins (Seo et al., 2006). Derivatives of neural crest cells in the pharyngeal arches and endocardial cushions of the cardiac outflow tract express Foxc2 (Gitler et al., 2004; Winnier et al., 1999). Foxc2 is also present in the kidneys and ovaries (organs of the urogenital system derived from the intermediate mesoderm) (Kume et al., 2000; Mattiske et al., 2006). In mesenchymal tissues giving rise to the cranial, appendicular, and axial skeleton, Foxc2 can be found in the early skeletal cell condensation and in C1 mesodermal cells (Nifuji et al., 2001). Additionally, Foxc2 is expressed in adipose tissue (Cederberg et al., 2001) and the lymphatic vasculature (Petrova et al., 2004).

The function of Foxc2 in development and tissue function has been explored through gene deletion and overexpression studies. Initial characterization of $Foxc2^{-/-}$ mice revealed skeletal, ocular, and cardiovascular defects (coarctation and interruption of the aortic arch) that led to perinatal death (Iida et al., 1997; Winnier et al., 1997). Renal defects (hypoplastic kidneys and abnormal glomerular development) have also been found in mice with Foxc2 deletion (Kume et al., 2000; Takemoto et al., 2006). Moreover, Foxc2 has been found to be a key regulator of metabolism. Foxc2 expression

in adipocytes is elevated in mice put on a high fat diet, and both global and adipocyte-specific overexpression of Foxc2 counteracts hypertriglyceridemia and diet-induced insulin resistance in mice (Cederberg et al., 2001; Kim et al., 2005). In terms of lymphatic development, loss of Foxc2 leads to abnormal mural cell recruitment to lymphatic capillaries and valve agenesis (Petrova et al., 2004). The above studies demonstrate a wide array of functions for Foxc2 during development and also point to its importance in postnatal metabolic processes.

*Based on current knowledge of protein expression profiles, it is interesting to note that Foxc2 and Cx37 are coexpressed in a number of the same tissues and cell types (i.e. blood and lymph endothelium, bone cells, granulosa cells) at various stages of life. In chapter 4, I will discuss the phenotype of $Foxc2^{+/-}Cx37^{-/-}$ mice which will serve to highlight the interdependence between Cx37 and Foxc2 during organogenesis.*

## Foxc2 in the lymphatic vasculature

One of the first indications that Foxc2 was involved in lymphatic development came from gene linkage studies done in patients with lymphedema-distichiasis (LD, OMIM #153400). LD is caused by mutations in Foxc2 and is characterized by lymphedema (that manifests at varying ages) and distichiasis (double row of eyelashes). The pleiotropic effects of Foxc2 are underlined by the clinical variability seen in patients with LD, which include problems such as extradural cysts, cardiac defects, varicose veins, cleft palate, photophobia, renal disease, and diabetes mellitus (Brice et al., 2002; Fang et al., 2000; Finegold et al., 2001; Yildirim-Toruner et al., 2004). In mice, haploinsufficiency of Foxc2 has been found to mimic the ocular (distichiasis) and lymphatic phenotype (lymph vessel and lymph node hyperplasia) of LD in humans (Kriederman et al., 2003). Foxc2 also figures prominently in lymphatic valve formation and vascular patterning, as $Foxc2^{-/-}$ embryonic mice have enlarged lymphatic vessels, inappropriate smooth muscle cell coverage of lymph capillaries, and develop edema.

The precise mechanisms by which Foxc2 controls lymphatic development are still being elucidated. Foxc2 has been found to regulate the expression (either directly or indirectly) of a number of genes involved in vascular development such as the

transmembrane receptors Notch1 and Notch4, the Notch ligands Hey2 and Dll4, Ang2, integrin-β3, C-X-C chemokine receptor type 4 (CXCR4), ephrin-B2, Vegf, and PDGF-β (Hayashi and Kume, 2009, 2008; Hayashi et al., 2008; Seo et al., 2006; Xue et al., 2008). In addition, $Foxc2^{+/-}Vegfr3^{+/-}$ mice have similar lymphatic defects compared to $Foxc2^{-/-}$ mice, a genetic interaction demonstrating that Foxc2 interacts with Vegfr3 signaling (Petrova et al., 2004). Foxc2 also interacts with another transcription factor, nuclear factor of activated T-cells c1 (Nfatc1, a gene that is involved in heart development), evinced by the identification of numerous cooperative binding sites in the human genome (Chang et al., 2004; Kumai et al., 2000; Norrmén et al., 2009). Recently, experiments done in cell culture have found that Cx37 is necessary for the coordinated activation of Nfatc1 in response to oscillatory shear stress. Therefore, Sabine et al. have proposed a mechanism whereby mechanotransduction, Cx37, Foxc2, and Nfatc1 coordinate the events that result in lymphatic valve formation (Sabine et al., 2012).

*Our characterization of $Foxc2^{+/-}Cx37^{-/-}$ mice (presented in chapter 4 and paper II) lends support to the model proposed by Sabine and colleagues. Furthermore, we demonstrate that Foxc2 function depends on Cx37 (either directly or indirectly) during lymphatic vascular growth and remodeling.*

# CHAPTER 2: CONNEXIN EXPRESSION IN THE LYMPHATIC VASCULATURE

Since the discovery of gap junctions and connexins in the middle of the 20th century, there have been relatively few studies done regarding these structures and proteins in the lymphatic vasculature. The standard for determining the presence of gap junctions between cells has been through either transmission electron microscopy (TEM) or via experiments that demonstrate functional (i.e. electrical or dye) coupling. Several studies employing TEM to determine the existence of gap junctions within the lymphatic vasculature were performed with mixed results. In the late 1970s, Rhodin and Sue evaluated the lymph vessels of the rat mesentery, but the data from their micrographs were ultimately inconclusive. Despite this, they proposed that gap junctions existed between lymphatic endothelial cells (Rhodin and Sue, 1979). Studies done by Compton and Raviola in the rabbit clearly demonstrated gap junctions between the sinus-lining endothelium of the lymph node via electron microscopy (Compton and Raviola, 1985). TEM micrographs also reportedly depict gap junctions between lymphatic smooth muscle cells (McHale and Meharg, 1992). In the smooth muscle cells of rat, canine, and feline thoracic duct, "gap junction-like" contacts have been described (Bannykh et al., 1994; Nakamura and Yamamoto, 1988). The expression of Cx43 was later demonstrated immunohistochemically in the sinus-lining endothelial cells of the lymph node from humans (Krenács and Rosendaal, 1995). Additionally, experiments using bath-applied, non-specific gap junction blockers (n-heptanol, oleic acid) in preparations of rat and bovine mesenteric collecting vessels resulted in decreased frequency of propagated contractions and disrupted contractile coordination (McHale and Meharg, 1992; Zawieja et al., 1993). Within the last decade, there has been renewed interest in studying connexins within the lymphatic vasculature. Differential gene expression between human LECs and BECs was evaluated through gene chip experiments, and Cx47 RNA was found in LECs but not BECs (Wick et al., 2007). Based on this finding, Ferrel et al. screened for mutations in the gene coding for Cx47 (GJC2) in human patients with lymphedema and identified multiple GJC2 missense mutations as causes of primary lymphedema (Ferrell et al., 2010). GJC2 mutations also increase the risk of developing

secondary lymphedema post breast cancer treatment, suggesting that Cx47 is important for reparative lymphangiogenesis (Finegold et al., 2012). Moreover, ectodermal deletion of Cx26 in mice results in defective peripheral lymphangiogenesis (Dicke et al., 2011).

The studies mentioned above formed the groundwork for exploring gap junctions and connexins within the lymphatic vasculature, but a great deal remains unknown. All in all, there is a limited amount of ultrastructural and physiologic data regarding gap junctions and their function in the lymphatic vasculature. The evidence so far seems to indicate that gap junctions exist between lymphatic smooth muscle cells (SMC), but their presence between LECs (or between LECs and lymphatic SMCs) has not been definitively determined. Nevertheless, aside from the identification of Cx43 protein in lymph node endothelium and Cx47 RNA in LECs from primary skin cultures, connexin expression in human lymphatic vessels has not been fully characterized. Functionally, gap junctions are likely important for the coordination of lymphatic SMCs in the lymphangion, but the dynamics of this regulation are still a mystery. Electrical conduction from lymphangion to lymphangion along the vessel is likely the best explanation. However, direct microelectrode recordings from actively pumping lymph vessels are precluded by the amount of tissue movement (alternatively, the use of suction electrodes results in abolishment of coordinated pumping) (McHale and Meharg, 1992).

Much of what *is* known about connexin expression and gap junction function in vascular beds is derived from numerous studies focused on the cardiovascular system. Of the 21 connexin isoforms in humans, Cx37, Cx40, Cx43, Cx45, and Cx47 have been characterized in the heart and/or blood vessels. Each of these connexins has a specific expression profile within the layers of the blood vessel wall, as well as differing distributions based on the level of the blood vasculature in question (i.e. arteries, capillaries, veins, etc.). Presumably, the differential expression of these connexins reflects the leveraging of their unique functional characteristics. The distinction between their roles in vascular development and function are highlighted by the phenotype of mice lacking specific connexin isoforms (either globally or in a tissue-specific manner). $Cx37^{-/-}$ mice lack venous valves (Munger et al., 2013). Mice with global deletion of $Cx43^{-/-}$ mice have obstruction of the pulmonary outflow tract whereas cardiac-specific deletion results in reduced ventricular conduction velocity and lethal spontaneous

arrhythmias (Gutstein et al., 2001; Munger et al., 2013). Ablation of Cx40 in mice results in impaired conduction of vasodilator responses and hypertension (de Wit et al., 2003, 2000). Mice with deletion of Cx45 die *in utero* of heart failure around E10 (Kumai et al., 2000). Interestingly, Cx47 knockout mice are overtly healthy, albeit with some vacuolation of nerve fibers in the central nervous system (Odermatt et al., 2003).

Thus, using the blood vasculature as a point of reference, it might be expected that the lymphatic vasculature would have some similar characteristics regarding the identity of connexins expressed as well as their distribution. With this in mind, we sought in the present study to investigate connexin expression throughout the hierarchy of the lymphatic vasculature at different stages of development. To accomplish this, we used immunohistochemistry (IHC) to evaluate lymphatics in tissues collected from mice at various stages of life – from embryonic to adult. We used antibodies directed against lymphatic endothelial cell markers (Prox1, Vegfr3, and Lyve1) to identify lymphatic vessels and antibodies against Cx26, Cx37, Cx40, Cx43, Cx45, and Cx47 to probe for connexin expression.

## Expression during embryonic and early postnatal time points

We found that both Cx37 and Cx43 were expressed in LECs of the jugular lymph sac (JLS), a structure that forms early in lymphatic vascular development (Figure 1A-E; I). Interestingly, Cx37 and Cx43 were usually expressed in distinct domains within the JLS, with areas of high Cx37 next to areas of low Cx43 and vice versa (high Cx43 next to low Cx37). Cx26, Cx40, Cx45, and Cx47 were not detected in the JLS at E13.5. Thus, even at this early stage of development (E12.5 – E13.5), differential expression of connexins was evident in the continuous layer of LECs in the JLS.

Later in development, Cx37 and Cx43 expression was found in LECs of various developing lymphatics at E16.5 and E18.5 (dermal, subcutaneous, mesenteric, submucosal, and thoracic duct) (Figure 1F-Q, 2A-G; I). Cx37 and Cx43 were also present in LECs of the lacteals and submucosal lymphatics of the colon (Figure S6; I, Figure 8; II). Cx47 was expressed at these time points as well, but it was restricted to a subset of LECs in collecting vessels (in the skin and mesentery). We did not detect Cx26

expression in the lymphatics during this period of embryonic development nor were we able to detect it during postnatal lymphatic development.

Fascinating changes in Cx37 and Cx43 expression within the endothelium occurred throughout maturation of the collecting vessels. Both of these connexins were coexpressed in the general endothelium of the mesenteric collectors, but became progressively enriched in the lymphatic valves during the transition from late embryonic stages to postnatal periods (Figure 2A-L; I). Amazingly, Cx37 and Cx43 expression was exquisitely separated between the two sides of the lymphatic valve leaflet (Figure 2J-K, I) – a difference that became more evident in adult valves. Cx47 continues to be expressed through this embryonic to postnatal transition, and its restriction to a select number of valve LECs was more apparent postnally (Figure 2M-P; I).

## Expression in the lymphatic vasculature: adulthood

In adult tissues, we found Cx37 and Cx43 in LECs of collecting vessels. Strikingly, in the valves of the thoracic duct, the separation in Cx37 and Cx43 between the valve leaflets was quite pronounced. Cx37 was highly expressed in LECs of the downstream leaflet, whereas Cx43 was highly expressed in LECs of the upstream leaflet (Figure 3A-H; I, also shown in schematic form in the figure of paper III). Cx47 was found in the thoracic duct valve leaflets in a restricted group of LECs, where it colocalized with Cx43 (Figure 3I-J, I). We did not detect Cx26, Cx40, Cx45, or Cx47 within non-valve LECs of the thoracic duct. Also, Cx37 and Cx43 were not expressed in LECs of the dermal lymphatic capillaries within the ear (Figure 3M-N, I).

## Summary and discussion

In mice, Cx37, Cx43, and Cx47 are expressed within the LECs of collecting vessels. Cx37 and Cx43 are coexpressed within the non-valve endothelium and tend to colocalize, but are enriched and differentially expressed on the downstream and upstream valve leaflets, respectively. Cx47 is found in a conspicuous subset of LECs. While Cx37 and Cx43 are expressed in the lacteals, they are absent in the dermal lymphatic capillaries of the adult ear. Thus, similar to the blood vasculature, connexins are expressed in

distinct domains/levels of the lymphatic vasculature. This differential expression likely underlies discrete roles for connexins in lymphatic vascular development and function.

The colocalization of Cx37, Cx43, and Cx47 in LECs raises the interesting possibility of heteromeric/heterotypic channel formation between cells of the lymphatic vasculature. Cx37 and Cx43 are capable of forming heteromeric and heterotypic gap junction channels in cell culture (Brink et al., 1997; Yeager and Nicholson, 2000), and it is possible that these types of channels form in non-valve endothelium. Cx47 and Cx43 are expressed in glial cells of the central nervous system and permit communication between oligodendrocytes (expressing Cx47) and astrocytes (expressing Cx43) through heterotypic channel formation (Magnotti et al., 2011). Therefore, Cx47/Cx43 heterotypic channel formation between endothelial cells of the valve should be considered as well (the figure in paper III illustrates possible channel permutations). Moreover, our data is the first report (to our knowledge) of Cx43 and Cx47 coexpression within an individual cell. Hence, the potential for Cx43/Cx47 heteromeric channel formation in LECs should be taken into account. While we found colocalization of these connexins through IHC, techniques with much higher spatial resolution will be needed to definitively determine the composition/arrangement of connexin-based channels in LECs.

# CHAPTER 3: STRUCTURAL AND FUNCTIONAL CONSEQUENCES OF CONNEXIN37 AND CONNEXIN43 DEFICIENCIES IN THE LYMPHATIC VASCULATURE

In previous studies of Cx37 and Cx43 knockout mice, no lymphatic defects were noted. As mentioned before, global deletion of Cx43 results in embryonic lethality, but Cx43 heterozygous mice ($Cx43^{+/-}$) are overtly normal (Reaume et al., 1995). $Cx37^{-/-}$ female mice are infertile, but no other obvious deleterious features were reported in their initial characterization (Simon et al., 1997). In order to investigate the role of Cx37 and Cx43 in the development and function of the lymphatic system, we generated mice with genetic deficiencies in Cx37 and/or Cx43. The morphology of lymphatic structures at embryonic and adult stages was evaluated through H&E and immunohistochemical staining of sections or whole-mount tissues. Evans blue dye (EBD) injections were also performed on adult mice to assess lymph flow. EBD binds tightly to proteins within the interstitial fluid, and its clearance through the lymphatics can be used trace lymph flow.

## Effects on embryonic lymphatic vascular development

Given the prominent expression of Cx37 and Cx43 in embryonic lymphatic structures, we expected that the loss of these two proteins would produce defects evident throughout various stages of development. Indeed, the JLS of $Cx37^{-/-}$, $Cx43^{-/-}$, and $Cx37^{-/-}Cx43^{-/-}$ mice was significantly enlarged at E13.5 (Figure 4A-C; I). Also, severe edema frequently manifested in $Cx37^{-/-}Cx43^{-/-}$ mice in late embryonic development (Figure 5A, B-G; I). Edema was sometimes found in $Cx37^{-/-}Cx43^{+/-}$ embryos, but was not usually observed in $Cx37^{-/-}$ or $Cx43^{-/-}$ embryos. At E18.5, the thoracic duct as well as the intercostal and diaphragmatic lymphatics were abnormally patterned and displayed erratic caliber in $Cx43^{-/-}$ and $Cx37^{-/-}Cx43^{-/-}$ mice (Figure 6A-H; I). In $Cx37^{+/-}Cx43^{-/-}$ and $Cx37^{-/-}Cx43^{-/-}$ embryos, blood was widely found in the dermal, thoracic, and intestinal lymphatics (Figure 7A-I; I).

One of the most striking attributes of $Cx43^{-/-}$ and $Cx37^{-/-}Cx43^{-/-}$ embryos was the complete absence of lymphatic valves within the mesentery (Figure 8A-G; I). Loss of Cx37 alone, however, resulted in a reduction (by approximately 50%) in the number of

developing valves in the mesenteric lymphatics and dermal lymphatics (Figure 8I; I and Figure 9T; III). There was also a reduction in valves within the mesenteric collecting vessels in $Cx37^{+/-}Cx43^{+/-}$ mice, though this reduction was less pronounced (Figure 8I; I). Dermal lymphatic valves were not present in the skin of $Cx37^{-/-}Cx43^{-/-}$ mice, but could still be found in $Cx37^{-/-}$ or $Cx43^{-/-}$ mice (albeit at reduced frequency).

## Lymphatic pathology in adulthood

The postnatal viability of $Cx37^{-/-}Cx43^{+/-}$ mice was reduced, and mice of this genotype died from chylothorax (leak of chyle into the thoracic cavity) at variable ages (Figure 9B; I). EBD injections performed on $Cx37^{-/-}Cx43^{+/-}$ mice revealed dye reflux in dermal lymph vessels of the ear and hindlimb, mesenteric lymph nodes, and intercostal lymphatics (Figure 9F-I, K, N; I). In some cases, EBD was not found in the thoracic duct following injection in the hindpaw of $Cx37^{-/-}Cx43^{+/-}$ mice, indicating severely impaired lymph flow. Dye reflux in the lymphatics was also frequently seen in $Cx37^{-/-}$ mice (Figure 9E, M; I), but only rarely in $Cx37^{+/-}Cx43^{+/-}$ mice. These abnormalities in lymph flow suggested impaired lymphatic valve function, and subsequent histological analysis of the thoracic duct in $Cx37^{-/-}Cx43^{+/-}$ and $Cx37^{-/-}$ demonstrated a significant reduction in valves (Figure 10A-E; I).

## Summary and discussion

Lymphatic vascular developmental defects occur in mice lacking Cx37 and/or Cx43. Consequences of their loss can be seen as early as E13.5 with enlargement of the JLS. Surprisingly, the development of lymphatic valves is disrupted when these connexins are absent. Cx43 is essential for the initiation of valve formation, as ablation of this connexin results in complete lymphatic valve agenesis in mesenteric collectors. However, some valves still form in the dermal lymphatics of $Cx43^{-/-}$ mice. This finding could be explained by a more prominent role for Cx43 in some types/mechanisms of valve formation (see Kampmeier, 1928) versus others. Additionally, differences in the relative expression levels of Cx43 in the dermis compared to the mesentery may be a contributing factor. While valve initiation still occurs in mesenteric and dermal

lymphatics of $Cx37^{-/-}$ mice, the number of valves is reduced. The preponderance of dye reflux in $Cx37^{-/-}$ mice suggests that the disturbance of valve formation during embryonic development has repercussions that extend into adulthood.[2] These data demonstrate that Cx37 is important for maturation/maintenance of lymphatic valves. Furthermore, combining loss of one copy of Cx43 with complete deletion of Cx37 results in more severe lymphatic dysfunction (as evinced by chylothorax and extensive dye reflux in $Cx37^{-/-}Cx43^{+/-}$ mice) than loss of Cx37 alone. It is likely that the exacerbation of valve deficiency in the thoracic duct (and elsewhere) of $Cx37^{-/-}Cx43^{+/-}$ mice contributes to this effect. The appearance of blood in the lymphatics of $Cx37^{+/-}Cx43^{-/-}$ and $Cx37^{-/-}Cx43^{-/-}$ *in utero* may be explained by lymphatic valve deficiencies allowing retrograde filling of blood from the venous circulation. Another possible explanation for lymph-blood mixing would be the formation of improper lymphatic-venous anastomoses, but we did not find morphological evidence for this. Indeed, it is unlikely that small, isolated lymphatic-venous connections account for the shear extent of blood found in the lymphatics of $Cx37^{-/-}Cx43^{-/-}$ mice. However, an exhaustive histological analysis would be required to unequivocally rule out this possibility.

[2] Intriguingly, valve deficiencies in $Cx37^{-/-}$ mice have gone unnoticed for many years, highlighting the fact that they are rather resilient to the loss of most lymphatic (and venous) valves. This may be due to a relatively low (compared to humans) potential hydrostatic pressure gradient from the peripheral to central vessels as a consequence of being small quadrupeds. For instance, there is a potential hydrostatic pressure gradient of approximately 110 mm Hg from the feet to the large cervical veins of an upright human (average height, ~1.7m) (Zawieja, 2009). Consider that the hydrostatic pressure of a column of fluid is directly proportional to its height and that mice are over 15-20 times "shorter" than adult humans. Thus, the presence of valves to segment this hydrostatic column is crucial to maintaining sufficient fluid flow in humans, but is likely less critical in mice.

# CHAPTER 4: FOXC2 AND CONNEXIN37 – MOLECULAR INTERDEPENDENCE IN LYMPHATIC VASCULAR DEVELOPMENT

Foxc2 mutations cause lymphedema-distichiasis in humans, and characterization of $Foxc2^{+/-}$ mice has shown them to be a relevant model for this disease (Fang et al., 2000; Kriederman et al., 2003). $Foxc2^{+/-}$ mice have incompetent lymphatic valves, and $Foxc2^{-/-}$ mice fail to form lymphatic valves (Kriederman et al., 2003; Petrova et al., 2004). These valve-related phenotypes prompted us to investigate the involvement of this transcription factor in regulating Cx37 and Cx43. To this end, IHC was used to probe for Cx37 and Cx43 expression in Foxc2 knockout mice. We also generated and characterized mice with combined genetic deficiencies in Foxc2 and Cx37 or Cx43. Lymphatic vessel architecture in the skin, mesentery, and intestine were evaluated using IHC and quantified by creating digital, skeletonized vessel networks to measure parameters such as vascular density, branch point density, and tortuosity.

## Lymphatic and craniofacial development

Cx37 expression was largely absent in the JLS of $Foxc2^{-/-}$ mice at E13.5, while it remained unaffected in nearby arteries. Conversely, Cx43 expression was still present in the JLS of $Foxc2^{-/-}$ mice (Figure 11A-D; I). In the mesenteric lymphatics at E17.5, expression of Cx37 was also reduced while arterial expression was unchanged (Figure 11E-F; I).

Because $Foxc2^{-/-}$ mice die *in utero*, we focused on characterizing mice with Cx37 and Cx43 deficiencies combined with Foxc2 haploinsufficiency. $Foxc2^{+/-}Cx37^{+/-}$ and $Foxc2^{+/-}Cx43^{+/-}$ mice survived into adulthood with no apparent reductions in viability. $Foxc2^{+/-}Cx37^{-/-}$ mice, however, died perinatally.

Several craniofacial and lymphatic defects manifested in $Foxc2^{+/-}Cx37^{-/-}$ mice by E18.5. $Foxc2^{+/-}Cx37^{-/-}$ mice had developed gross morphological changes to the shape of the face. These embryos frequently had blunted snouts and an undersized jaw (micrognathism, Figure 1A-E; II). Generalized edema was also detected in some cases (Figure 1F-J). Morphometric measurements of the head confirmed blunting of the snout

in these animals compared to other genotypes (Figure 2A-F; II). Like $Cx37^{+/-}Cx43^{-/-}$ and $Cx37^{-/-}Cx43^{-/-}$ embryos, blood was found in the lymphatics of $Foxc2^{+/-}Cx37^{-/-}$ mice. Curiously, in $Foxc2^{+/-}Cx37^{-/-}$ mice, these regions were limited to certain locations – dermal lymphatics in the torso, submucosal lymphatics of the small intestine, and pericardial lymphatics (Figure 3A-K; II).

Analysis of the lymphatic networks at E18.5 in the intestine, mesentery, and skin of $Foxc2^{+/-}Cx37^{-/-}$ mice via IHC revealed several striking changes. The submucosal lymphatics in the small intestine of $Foxc2^{+/-}Cx37^{-/-}$ mice were extremely dilated (lymphangiectasia) and had erratic caliber (Figure 4A, C, E, G; II), while those of the large intestine were not appreciably affected (Figure 4B, D, F, H; II). The submucosal lymphatics had larger vessel diameters, higher vascular density, and fewer branch points (Figure 4I-L; II). Moreover, the lacteals of these mice were significantly shorter (Figure 5A-J; II). In the mesentery, lymphatic collectors were cavernous and failed to remodel into a branched network. Based on previous results indicating effects on cell growth with the loss of Cx37 alone (enlarged JLS), we measured mitotic index (using the mitosis-specific marker phospho-histone H3) and found an increase in mesenteric LEC proliferation in $Cx37^{-/-}$ mice (Figure 7A-I; II). Additionally, there was agenesis of valves in $Foxc2^{+/-}Cx37^{-/-}$ mice, with no indication of early valve initiation (Figure 6A-P; II). The dermal lymphatics also completely lacked valves. Surprisingly, contrary to the enlargement seen in the intestinal and mesenteric lymphatics, the dermal lymphatics had a smaller, erratic caliber (Figure 9A-L; II). Despite this general reduction in lymph vessel size, some areas within the skin of $Foxc2^{+/-}Cx37^{-/-}$ embryos developed large structures that resembled cystic lymphangiomas (Figure 9M-O; II).

## Lymph flow in $Foxc2^{+/-}Cx37^{+/-}$ and $Foxc2^{+/-}Cx43^{+/-}$ adult mice

Due to the perinatal lethality of $Foxc2^{+/-}Cx37^{-/-}$ mice, we could not evaluate adult mice of this genotype. $Foxc2^{+/-}Cx37^{+/-}$ mice are viable into adulthood, though. EBD injections did not reveal any overt differences in dye clearance for $Foxc2^{+/-}Cx37^{+/-}$ mice. Since mice with deletion of Cx43 lack lymphatic valves, we also explored dye clearance

in $Foxc2^{+/-}Cx43^{+/-}$ adult mice. Similar EBD patterns were seen in the lymphatics of $Foxc2^{+/-}Cx43^{+/-}$ mice compared to other genotypes (Figure 10A-L; II).

## Summary and discussion

Our results demonstrate that Cx37, but not Cx43, expression is controlled either directly or indirectly by Foxc2. The phenotype of $Foxc2^{+/-}Cx37^{-/-}$ mice reveals that these proteins are critical for craniofacial morphogenesis as well as lymphatic vessel growth, patterning, and remodeling. Interestingly, region-specific changes in mice of this genotype (i.e. lymphangiectasia in the intestine and mesentery versus diminished dermal lymphatic caliber) suggest that Foxc2 and Cx37 have different roles in lymphatic development depending on the particular lymph-vascular bed in question. Consistent with reports that Cx37 serves to decrease proliferation in cell culture (Burt et al., 2008), loss of Cx37 increases the mitotic activity of LECs. This effect may partly explain the hyperplasia seen in some lymphatics and underlie the formation of lymphangiomas in the skin of $Foxc2^{+/-}Cx37^{-/-}$ mice.

While the specific processes leading to lymphatic valve development remain unclear, Sabine et al. proposed a mechanism whereby mechanotransduction, Cx37, Foxc2, and Nfatc1 direct the cellular activities that result in valvulogenesis (Sabine et al., 2012). In their model, Cx37 is required for the calcium-dependent, coordinated activation of Nfatc1 and its translocation into the nucleus. Foxc2 and Nfatc1, either cooperatively or separately, then regulate the expression of genes that set into motion the events that guide valve formation. The loss of lymphatic valves in $Foxc2^{+/-}Cx37^{-/-}$ mice can also be explained through this model. In response to mechanical sheer stresses due to turbulent lymph flow, decreased intercellular communication as a result of Cx37 absence leads to reduced calcium signaling between LECs. Uncoordinated/inefficient activation of Nfatc1, in addition to a prima facie reduction in levels of Foxc2, then causes insufficient expression of the genes necessary to initiate the valve program. Regardless, the lymphatic derangements in $Foxc2^{+/-}Cx37^{-/-}$ embryos (which are not seen in $Foxc2^{+/-}$ or $Cx37^{-/-}$ mice separately), suggest that Foxc2 function relies on Cx37. Whether this

function (*in vivo*) hinges on channel-dependent or channel-independent roles of Cx37 remains to be determined.

The fact that Foxc2$^{+/-}$Cx37$^{-/-}$ mice developed a spectrum of defects including primary lymphangiectasia, lymphedema, and craniofacial abnormalities is rather intriguing. Hennekam syndrome is a hereditary lymphatic disorder in humans that is characterized by the concurrent presentation of these disease features. Mutations in Ccbe1 (collagen and calcium-binding EGF domains 1) have been found to cause Hennekam syndrome in some, but not all, human cases. Considering mutations in Foxc2 and/or Cx37 may help identity the genetic basis of Hennekam syndrome (or other diseases that share this gallery of pathological changes) in which no known mutations have been found.

*A summary of the phenotypes we observed in Foxc2$^{+/-}$, Cx37$^{-/-}$, and Foxc2$^{+/-}$Cx37$^{-/-}$ mice is provided below.*

| | WT | Cx37-/- | Foxc2+/- | Foxc2+/- Cx37-/- |
|---|---|---|---|---|
| Viability | Adult | Adult | Adult | Perinatal death |
| Craniofacial morphology | Normal appearance | Normal appearance | Normal appearance | Blunted snout, micrognathia |
| Edema | Not observed | Embryonic, frequency < 5% | Adult, low frequency | In utero |
| Lymph-blood mixing | Not observed | Affecting skin**, frequency < 5% | Not observed | Affecting skin and intestine, frequency > 90% |
| Collecting lymphatics | Normal appearance | Mild enlargement | Hyperplasia | Severe lymphangiectasia |
| Lymphatic valves | Normal appearance | Reduced | Reduced | Absent |
| Dermal lymphatics | Normal appearance | Normal appearance | Reduced caliber | Reduced caliber, discrete lymphangiomas |
| Lacteals | Normal caliber, spans each villus | Normal caliber, spans each villus | Normal caliber, spans each villus | Reduced length (spans ~1/2 the villus) |

**Figure 4.1 –** Summary of phenotypic features in mice with deficiencies in Foxc2 and/or Cx37. Genotypes are listed across the top, specific features are listed along the left side. Color scale is a subjective representation of the consequence of a given feature, ranging from blue (innocuous) to reddish brown (harmful).

# CHAPTER 5: CONCLUDING REMARKS AND FUTURE DIRECTIONS

> "The scientist is a practical man and his are practical (i.e., practically attainable) aims. He does not seek the **ultimate** but the **proximate**. He does not speak of the last analysis but rather of the next approximation. His are not those beautiful structures so delicately designed that a single flaw may cause the collapse of the whole. The scientist builds slowly and with a gross but solid kind of masonry. If dissatisfied with any of his work, even if it be near the very foundations, he can replace that part without damage to the remainder. On the whole he is satisfied with his work, for while science may never be wholly right it certainly is never wholly wrong; and it seems to be improving from decade to decade."
>
> *G. N. Lewis. Quoted in **Stochiometry** by Leonard K. Nash. Addison-Wesley 1966. p. vii.*

I feel a powerful resonation with what Gilbert Lewis said above. While the blocks of knowledge that I have laid down are by no means as significant as those placed by him, I nonetheless see them as important stones atop a greater structure representing the understanding of lymphatic development. With the help and guidance of my mentor and colleagues, this undertaking has formed the foundation for my first personal venture into scientific research. However, the work is seemingly always incomplete. Previous, unresolved questions linger in the back of our minds and new questions lurk around the corner. As for the *proximate*, I present below a few different avenues for future investigations.

### *Gap junction communication between cells comprising the lymph vessel*

One critical question has still not been definitively resolved – where are the gap junctions in the lymph vessel wall? The disruption of lymph vessel coordination in preparations bathed in the non-specific gap junction inhibitors n-heptanol and oleic acid suggested that gap junction communication was important for lymphangion contractions (McHale and Meharg, 1992; Zawieja et al., 1993). In addition to this, various studies have shown gap junctions between SMCs of the thoracic duct in different species (Bannykh et al., 1994; Nakamura and Yamamoto, 1988), but there is little evidence for gap junctions between LECs. The best indication so far has been the demonstration of gap junctions between the sinus-lining endothelium of lymph nodes (Krenács and

Rosendaal, 1995); however, the lymph nodes are quite distinct from the lymph vessels. Furthermore, myoendothelial gap junctions (MEGJs) have been described as a form of heterocellular communication (Isakson and Duling, 2005). Assuming gap junctions are between LECs, are there also MEGJs in the lymphatic vessel? Additionally, given the changes in connexin expression in the lymphatic endothelium throughout embryonic development, how does the nature of gap junction coupling change as a consequence?

Connexin expression and function in lymphatic SMCs were aspects we did not exhaustively explore. Recent work by Lutter et al. demonstrated that SMC recruitment to the dermal lymphatics of the ear did occur until approximately P14 (Lutter et al., 2012). Connexins may influence this recruitment process and be a potential factor involved in impaired lymphatic function seen in connexin-deficient mice. Moreover, the loss of connexins may alter coupling in distinct ways to affect lymph vessel contractility. In the blood vascular microcirculation of mice, endothelial cell coupling is critical for transmitting a dilatory signal (Emerson and Segal, 2000; Looft-Wilson et al., 2004) whereas conducted vasoconstrictions travel through the vascular SMC layer (Budel et al., 2003). Differential connexin expression between LECs and lymphatic SMCs may use a similar mechanism to control lymph vessel tone.

These rifts in knowledge demonstrate a need to further characterize connexin expression in the lymphatics of wild-type animals through IHC. Also, the presence of gaps junctions remains to be investigated between LECs and SMCs at different stages of life either via ultrastructural studies or assessment of functional coupling (through electrical recording or intracellular dye injection in isolated lymphatics). Scallan and Davis recently developed a technique to isolate and cannulate mouse popliteal lymphatic vessels (Scallan and Davis, 2013). Using their technique to record the contractile activity of popliteal lymphatic vessels in the presence or absence of more specific gap junction inhibitors (such as connexin-mimetic peptides) may be another experimental strategy to assess gap junction coupling.

### *LEC versus non-LEC dependent connexin expression in lymphatic development*

One of the weaknesses of using a global knockout mouse model is that the precise cell types that are responsible for the phenotype observed due to gene deletion cannot be

definitively identified. While the loss of Cx37 and/or Cx43 in LECs likely factors prominently in causing the lymphatic developmental abnormalities we observed, non-LEC expression of these connexins may be important as well. Macrophages "chaperone" tip cells as vessel growth occurs. Indeed, macrophages have been found to be important for defining dermal lymphatic caliber (Gordon et al., 2010). Interestingly, ATP release from Cx37-expressing macrophages inhibits leukocyte adhesion and has been suggested to be protective against atherosclerosis (Wong et al., 2006). Cx37 hemichannel function in macrophages may therefore be a mechanism of regulating lymphangiogenesis. Cx37 has also been found to be important in platelet function (Angelillo-Scherrer et al., 2011; Vaiyapuri et al., 2012), and a number of studies have provided compelling evidence for the role of platelets in proper lymph-blood separation during development (Bertozzi et al., 2010; Finney et al., 2012; Hess et al., 2014; Uhrin et al., 2010). Thus, lymph-blood mixing in $Cx37^{-/-}Cx43^{-/-}$ and $Foxc2^{+/-}Cx37^{-/-}$ mice might stem in part from the loss of Cx37 in megakaryocytes/platelets.

Generation of tissue-specific connexin knockout mice will aid in discriminating the principal players directing lymphatic development. One of the first steps will be to verify if deletion of Cx37 and/or Cx43 from LECs recapitulates the phenotype seen in global knockout of these genes. LEC-specific deletion could be achieved by crossing mice that express Cre recombinase under the control of a lymphatic-specific promoter (such as those for Prox1 or podoplanin) with mice that have loxP sites flanking the Cx37 or Cx43 gene. Additionally, using a similar approach to evaluate lymphatic morphogenesis in mice with targeted deletion of Cx37 from macrophages (Cre expression driven by the CD68 promoter) or platelets (Cre expression driven by the GPIbα promoter) may be other promising avenues of research.

### *Postnatal evaluation of $Foxc2^{+/-}Cx37^{-/-}$ mice*

Clinically, intestinal lymphangiectasia can give rise to chronic diarrhea, loss of serum proteins into the intestinal lumen (protein-losing enteropathy), and impaired absorption of fats and fat-soluble vitamins. These complications are believed to be primarily due to reduced lymph flow within the intestinal and mesenteric lymphatic networks. Failure to resolve the disruptions in intestinal lymphatic development seen in

$Foxc2^{+/-}Cx37^{-/-}$ mice would be expected to result in similar consequences. Postnatal lipid transport via lymphatics is critical to survival, and mice with defects in chylomicron uptake by lacteals die from wasting/starvation, with only 15% living past the first week of life, and less than 5% surviving to weaning age (Van Dyck et al., 2007). Problems related to intestinal loss of serum proteins and nutrient malabsorption during critical postnatal periods were likely major contributing factors to the death of $Foxc2^{+/-}Cx37^{-/-}$ mice. Medium-chain fatty acids (MCFAs) are often given to patients with intestinal lymphangiectasia, because unlike long-chain fatty acids (LCFAs), these shorter lipids can bypass the lymphatic vasculature and proceed directly to the blood. Though LCFAs represent the major lipid component of mother's milk, nutritive supplementation with MCFAs to alter the lipid profile of the mother's milk (or directly administered to the pups themselves) may increase the viability of $Foxc2^{+/-}Cx37^{-/-}$ mice and allow for detailed study of postnatal lymphatic development in these animals.

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# APPENDIX A: CONNEXIN37 AND CONNEXIN43 DEFICIENCIES IN MICE DISRUPT LYMPHATIC VALVE DEVELOPMENT AND RESULT IN LYMPHATIC DISORDERS INCLUDING LYMPHEDEMA AND CHYLOTHORAX

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# Connexin37 and Connexin43 deficiencies in mice disrupt lymphatic valve development and result in lymphatic disorders including lymphedema and chylothorax

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ABSTRACT

Intraluminal valves are required for the proper function of lymphatic collecting vessels and large lymphatic trunks like the thoracic duct. Despite recent progress in the study of lymphvasculogenesis and lymphangiogenesis, the molecular mechanisms controlling the morphogenesis of lymphatic valves remain poorly understood. Here, we report that gap junction proteins, or connexins (Cxs), are required for lymphatic valvulogenesis. Cx37 and Cx43 are expressed early in mouse lymphatic development in the jugular lymph sacs, and later in development these Cxs become enriched and differentially expressed by lymphatic endothelial cells on the upstream and downstream sides of the valves. Specific deficiencies of Cx37 and Cx43 alone or in combination result in defective valve formation in lymphatic collecting vessels, lymphedema, and chylothorax. We also show that Cx37 regulates jugular lymph sac size and that both Cx37 and Cx43 are required for normal thoracic duct development, including valve formation. Another Cx family member, Cx47, whose human analog is mutated in some families with lymphedema, is also highly enriched in a subset of endothelial cells in lymphatic valves. Mechanistically, we present data from Foxc2−/− embryos suggesting that Cx37 may be a target of regulation by Foxc2, a transcription factor that is mutated in human lymphedema–distichiasis syndrome. These results show that at least three Cxs are expressed in the developing lymphatic vasculature and, when defective, are associated with clinically manifest lymphatic disorders in mice and man.



## Introduction

Lymphatic (Ly) vessels are essential for tissue fluid balance, immune function, and the absorption and transport of dietary fat. Excess interstitial fluid is taken up by Ly capillaries and transported as lymph through valved collecting vessels, filtered by lymph nodes, and then carried through major Ly trunks which empty into the venous system. Valves are a crucial feature of the Ly vasculature because they ensure that lymph movement continues anterograde, propelled in part by Ly intrinsic contractions (Zawieja, 2009). Defects of the Ly system lead to a number of congenital and acquired disorders and syndromes including lymphedema, chylothorax, metabolic disorders, inflammation, and immune dysfunction. In addition, the Ly vasculature is a major route for tumor metastasis. Understanding the molecular mechanisms of lymphangiogenesis and lymphvasculogenesis both in normal contexts and during tumor growth will be important in efforts to develop novel molecular therapeutics (Jurisic and Detmar, 2009; Witte et al., 2001).

In recent years substantial progress has been made in identifying key genes and proteins involved in Ly development. Knockout and transgenic mouse models as well as other approaches have established the importance of key transcription factors (Prox1, Foxc2, Sox18, NFATc1, Coup-TFII, Net); signaling proteins (VEGF-C/D, Ang1 and Ang2, FIAF, EphrinB2, podoplanin, Syk, Akt/PKB (Zhou et al., 2010), PI3K); receptors (VEGFR-3, Np2); and cell-matrix interactions (integrin-α9, FnEIIIA, Emilin1) in Ly development (for reviews see (Mäkinen et al., 2007; Oliver and Srinivasan, 2010; Oliver and Srinivasan, 2008; Tammela and Alitalo, 2010)). In addition, several congenital Ly diseases have been linked to gene mutations, including Milroy disease (*VEGFR3*) (Ferrell et al., 1998), lymphedema–distichiasis syndrome (*FOXC2*) (Fang et al., 2000), hypotrichosis–lymphedema–telangiectasia (*SOX18*) (Irrthum et al., 2003), congenital chylothorax (*ITGA9*) (Ma et al., 2008), and generalized Ly dysplasia (*CCBE1*) (Connell et al., 2010). Despite these advances, the

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overall process of Ly development is still incompletely understood. The current view is that the process begins at E9.5-E10.5 in the mouse when a subgroup of committed Prox1-expressing endothelial cells in the anterior cardinal veins migrates laterally and form the Ly primordia (lymph sacs) (Oliver and Srinivasan, 2010). Primitive Ly capillary networks assemble by a process of centrifugal sprouting from the lymph sacs, and then subsequently these networks combine and remodel into a hierarchal network of initial and collecting Ly vessels (Tammela and Alitalo, 2010). However, detailed knowledge about the signaling mechanisms that govern these processes, and in particular, those that control Ly valve morphogenesis is at an early stage.

One area that has not been investigated in any detail is the role of gap junction (GJ) proteins, or connexins (Cxs), in Ly vascular development and function. Cxs are a family of 21 proteins in humans which assemble into GJ channels, structures that allow for the direct transfer of small molecules between adjacent cells (Goodenough and Paul, 2009). GJ channels are dynamically regulated, rapidly at the single channel level, and on a slower timescale at the level of Cx synthesis, assembly, post-translational modification, and degradation (Laird, 2006; Solan and Lampe, 2009). Besides intercellular channels, Cxs can also form hemichannels (undocked channels) which act as release sites for extracellular signaling molecules (Stout et al., 2004). Some Cxs bind other proteins within the cell, contributing to signaling that may be unrelated to channel function (Dbouk et al., 2009; Jiang and Gu, 2005; Laird, 2010). It is well established that endothelial cells and smooth muscle cells of many blood vessels are coupled by Cx-comprised intercellular channels (Gabriels and Paul, 1998; Simon and McWhorter, 2003; Yeh et al., 1997). Furthermore, Cxs have been shown to be necessary for various aspects of blood vessel development, propagation of conducted arteriolar vasomotor responses, and for communicating antiinflammatory signals between blood vessel endothelial cells (Brisset et al., 2009; Figueroa and Duling, 2009; Simon and McWhorter, 2002; Walker et al., 2005). Regarding Ly vessels, it has been suggested that GJs could provide a pathway for conduction of spontaneously evoked contractions in Ly vessels (McHale and Meharg, 1992; Zawieja et al., 1993). To date, however, the argument for GJs in Ly vessels is based mainly on GJ inhibitor studies. Because the inhibitors used were not specific for GJ channels, the evidence for GJs in Ly vessels has not been conclusive. While recent microarray studies of human dermal Ly endothelial cells (LECs) have provided evidence of expression of Cx mRNA in cultured LECs, the expression and localization of Cx proteins in developing and mature Ly vessels, as well as their potential functions in these vessels, have not been investigated (Shin et al., 2008; Wick et al., 2007). In this study, we examine the expression of Cxs in both the developing and mature Ly vasculature and use Cx-deficient mice to investigate their functions in the Ly system.

## Materials and methods

### *Mice and genotyping*

Cx37−/− (*Gja4*−/−) (Simon et al., 1997), Cx40−/− (*Gja5*−/−) (Simon et al., 1998), Cx43−/− (*Gja1*−/−) (Reaume et al., 1995), and Foxc2−/− (Iida et al., 1997) mice have been described previously. Cx37−/− and Cx43+/− mice were interbred to generate mice deficient in both Cxs. Mice were maintained on a C57BL/6 background and genotyped by PCR using previously published protocols for Cx40−/− (Simon and McWhorter, 2002), Cx43−/− (Bobbie et al., 2010) and Foxc2−/− lines (Kriederman et al., 2003). Primers for Cx37−/− genotyping were: Primer 1: 5′-GATCTCTCGTGGGATCATTG-3′; Primer 2: 5′-TGCTAGACCAGGTCCAGGAAC-3′; and Primer 3: 5′-GTCCCTTCG-TGCCTTTATCTC-3′. Animal protocols were approved by the IACUC Committee at the University of Arizona (Tucson, AZ).

### *Antibodies*

Primary antibodies used for immunostaining were as follows: rabbit antibodies to Cx37 (Simon et al., 2006), Cx37 (40–4200, Invitrogen), Cx40 (Gabriels and Paul, 1998), Cx43 (C6219, Sigma), Cx47 (36–4700, Invitrogen), LYVE-1 (ab14917, Abcam), NG2 condroitin sulfate (AB5320, Millipore), Prox1 (ab11941, Abcam); mouse antibodies to Cx26 (a gift from Paul Lampe), Cx43 (35–5000, Invitrogen), smooth muscle actin (C6198, Sigma), NFATc1 (sc-7294, Santa Cruz Biotechnology); rat antibodies to CD31 (MON1149, Cell Sciences), CD45 (550539, BD Biosciences), F4/80 (MF48000, Invitrogen), LYVE-1 (53–0443, eBioscience); goat antibodies to ephrinB2 (AF496, R&D Systems), Foxc2 (ab5060, Abcam), integrin-α9 (AF3827, R&D Systems), Prox1 (ab11941, Abcam), VEGFR-3 (AF743, R&D Systems); and hamster antibodies to CD3e (550275, BD Biosciences). AffiniPure minimal cross reactivity secondary antibodies (conjugated to Cy3, Cy5, or Dylight649) and unlabeled Fab fragments were from Jackson ImmunoResearch, AlexaFluor 488 goat anti-rat IgG from Invitrogen, and Vectastain Elite ABC kit (Rabbit IgG) from Vector Laboratories.

### *Section immunostaining*

Tissues were frozen unfixed in Tissue-tek O.C.T. and sectioned at 10 μm. Sections were fixed in acetone at −20 °C for 10 min, blocked in a solution containing PBS, 4% fish skin gelatin, either 1% goat serum or 1% donkey serum, 0.25% Triton X-100, and incubated with primary antibodies for 2 h. Sections were washed with PBS containing 0.25% Triton X-100 and then incubated with secondary antibodies for 30 min. After washing, sections were mounted in Mowiol 40–88 (Aldrich) containing 1,4-diazobicyclo-(2,2,2)-octane and viewed with an Olympus BX51 fluorescence microscope fitted with a Photometrics CoolSnap ES2 camera or viewed with a Zeiss LSM 510 confocal microscope. Ly vessels were identified by staining with antibodies against Prox1, VEGFR-3, or LYVE-1.

### *Whole-mount immunostaining*

Mesentery was fixed in 1% paraformaldehyde overnight at 4 °C, washed in PBS, permeabilized with PBS containing 0.3% Triton X-100, and then blocked overnight in PBS containing 3% goat serum and 0.3% Triton X-100. Primary antibodies diluted in PBS containing 0.3% Triton X-100 were applied to the tissue overnight at 4 °C. After washing, fluorescently labeled secondary antibodies were incubated overnight at 4 °C. Following final washes, the mesenteries were mounted on slides in Citifluor mountant (Electron Microscopy Sciences). Ear tissue was treated similarly except fixation was for 1 h at room temperature. For whole-mount Prox1 immunostaining of the TD and diaphragm muscle from embryos, the procedure was the same as with mesentery except Vectastain Elite ABC kit secondary and tertiary reagents and DAB substrate were used.

### *Lymphangiography with Evans blue dye*

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital and kept warm. Evans blue dye (EBD) (1% w/v) was injected intradermally into the hindpaws and a dissecting microscope was used to examine EBD transport. Evidence of abnormal dye reflux into hindlimb skin or mesenteric lymph nodes was noted if present. The thoracic cavity was then opened and the presence of EBD in the TD was noted along with any abnormal dye reflux into intercostal Ly vessels. In some mice, EBD was also serially injected into forepaws, snout, or ear to examine EBD movement in axillary, jugular, and ear regions, respectively.

*Quantification of thoracic duct valves*

Following EBD lymphangiography, the TD was dissected out, still attached to the aorta, from just above the diaphragm muscle to the top of the heart. The tissue was frozen unfixed in Tissue-tek O.C.T. and 10 μm serial sections (~750 sections/sample) were collected along the length of the TD. The number of valves per TD was determined from the serial sections. The position of the TD on the sections was determined by the location of EBD in the cryoblock face. Selected sections from the series were haematoxylin and eosin (H&E) stained or immunostained for Prox1 to further confirm the identification of the TD.

*Chyle analysis*

Chyle samples were submitted to the University of Arizona Animal Care Pathology Services Laboratory for determination of total protein, cholesterol, triglycerides and white cell counts. Triglyceride measurements were performed at Antech Diagnostics.

*Quantification of mesenteric valves*

Mesenteries from E18.5 embryos, cut into 3–4 segments, were whole-mount immunostained for Prox1 and CD31 as described above. The number of Ly valves per mesentery was determined by counting regions of locally elevated Prox1 staining, by an observer blind to the genotype. The CD31 co-staining pattern was used to confirm the identification of Ly valves.

*Quantification of jugular lymph sac (JLS) cross-sectional area and 3D volume reconstructions*

Embryos were collected at E13.5, embedded in paraffin, serially sectioned transversely (6 μm sections), and stained with H&E. The lumenal areas of the right and left JLS were measured at the point where the cervical nerve crosses the JLS. Images were taken using an Olympus microscope (BX51) equipped with a CCD camera and analyzed using Image-Pro Plus software. The numbers of animals analyzed for each genotype were: WT (7); Cx43−/− (4); Cx37−/− (6); and Cx37−/−Cx43−/− (6). Student's *t*-test (unpaired, unequal variance) was used for statistics. For 3D volume reconstructions, coronal sections were imaged at low magnification and stitched together with Photoshop CS4. Serial images were manually aligned and imported into BioImageXD for volume rendering. Volume estimates were determined using the Cavalieri method as described by Howard and Reed (Howard, 2005).

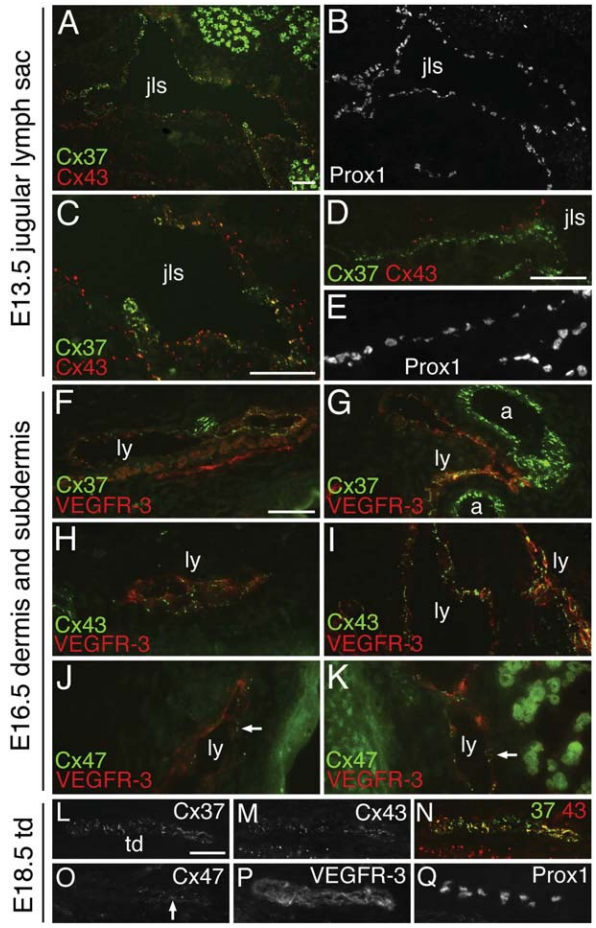


**Fig. 1.** Expression of Cx37, Cx43, and Cx47 in developing lymphatic vessels of the wild-type mouse embryo. (A–E) Immunolabeling of jugular lymph sac (jls) of E13.5 WT mouse embryos. Cx37 (green) and Cx43 (red) are differentially expressed in distinct domains in the JLS. The Cx37 antibody crossreacts with some muscle fiber types (upper right and lower right in panel A). (C) A higher magnification view of the JLS in (A). (D) Cx37 expression in this area is associated with a sprouting region of the JLS. (B and E) Prox1 labeling of sections adjacent to those in (A and D), respectively. (F–K) Immunolabeling of dermis and subdermis of E16.5 WT embryos. Cx37 and Cx43 (both green) are present in dermal lymphatics (ly) (F and H) as well as in deeper subcutaneous lymphatics (G and I). Cx37 is also highly expressed in arteries (a). Cx47 is weakly detected (arrows) in some E16.5 dermal (J) and subcutaneous lymphatics (K). (L–Q) Immunolabeling of thoracic duct (td) cross sections of E18.5 WT embryos. Cx37 and Cx43 are both present in the E18.5 TD (the TD lumen is collapsed in these sections) and there is significant colocalization of the Cx immunosignals (N). (O) Cx47 is weakly detected (arrow) in the E18.5 TD. (P and Q) VEGFR-3 and Prox1 labeling of adjacent sections to those in (L–O). Scale bars: (A and B) 50 μm; (C) 50 μm; (D and E) 50 μm; (F–K) 50 μm; (L–Q) 25 μm.

## Results

*Cx37, Cx43, and Cx47 are expressed in the developing lymphatic vasculature and become progressively enriched at lymphatic valves*

We looked for expression of vascular Cxs in Ly vessels during mouse embryonic development by immunofluorescence, starting with the jugular lymph sac (JLS), an early Ly primordium. At E12.5, Cx43 was already evident in the endothelium of the JLS but Cx37 expression was sparse. By E13.5, Cx37 and Cx43 were both clearly present in the JLS (Fig. 1A and C), whereas two other Cxs, Cx40 and Cx45, were not detected. Within the JLS, Cx37 and Cx43 were typically differentially expressed in distinct domains, with regions of high Cx37/low Cx43 expression next to regions of high Cx43/low Cx37 expression. Segregation of Cx37 or Cx43 expression was sometimes associated with extensions or sprouting regions of the JLS (Fig. 1D). Cx26, which is associated with Ly vessel invasion in breast cancer (Naoi et al., 2007), and Cx47, which is mutated in some families with primary lymphedema (Ferrell et al., 2010), were not detected in the E13.5 JLS.

At E16.5, Cx37 and Cx43 were present in dermal lymphatics of the skin as well as in deeper subcutaneous lymphatics (Fig. 1F–I), which tended to show higher Cx expression than superficial lymphatics strongly positive for VEGFR3 or LYVE-1. Cx47 was weakly detected in some of the E16.5 dermal and subcutaneous lymphatics (Fig. 1J and K) whereas Cx26 was not detected. At E18.5, Cx37 and Cx43 were both present in the Ly endothelium of the TD (Fig. 1L–N), and Cx47 was weakly detected there as well (Fig. 1O). Cx37 and Cx43 were also detected in other central Ly trunks at E18.5.

Cx37 and Cx43 were also prominently expressed in the developing mesenteric collecting lymphatics at E18.5. Ly vessels in the small intestine and its associated mesentery are essential for the absorption and transport of dietary fat from the intestine to the TD. Cx37 was also expressed in the endothelium of mesenteric arteries but not

mesenteric veins. Cx43 was not detected in the endothelium of either mesenteric arteries or veins. Whole-mount immunostaining revealed that Cx37 (Fig. 2A and E) and Cx43 (Fig. 2C and F) were concentrated at cell–cell interfaces throughout the endothelium of the collecting lymphatics, including at the intraluminal valves. Double labeling showed that Cx37 and Cx43 were colocalized in the endothelium of the collecting lymphatics (Fig. 2G) (except in valves, as discussed below). In the intestinal wall, Cx43 but not Cx37 was detected in the submucosal lymphatics and lacteals (see Fig. S6 in the supplemental material). Cx37 and Cx43 were also detected in E16.5 mesenteric collecting lymphatics and trace amounts of Cx37 were observed as early as E15.5, but not earlier (not shown). Postnatally, at P3–P7, Cx37 expression (Fig. 2H) became progressively enriched in the valves of the collecting lymphatics whereas Cx43 (Fig. 2I) remained uniformly expressed in the Ly endothelium, including valves. In the collecting lymphatics of 3–4 week old mice, Cx37 was highly enriched in the valves (Fig. 2J). Cx43 was also concentrated in valves at this age, although the degree of enrichment was more variable (Fig. 2J). Surprisingly, double labeling showed that Cx37 and Cx43 were remarkably differentially localized in the mesenteric valve leaflets (Fig. 2J and K) but not in the non-valve endothelium (Fig. 2L) (discussed further below).

In addition to Cx37 and Cx43, we also detected Cx47 in a subset of LECs in E18.5 mesenteric collecting lymphatics (Fig. 2M). At P3, Cx47 expression remained patchy in mesenteric lymphatics and was associated with valve leaflets (Fig. 2N). In the 3–4 week old mouse, Cx47 expression was highly restricted to a subset of cells within the valve leaflets of larger mesenteric collecting lymphatics and was often found near the base of the leaflet (Fig. 2O). Cx47 colocalized with Cx43 in those areas (Fig. 2P).

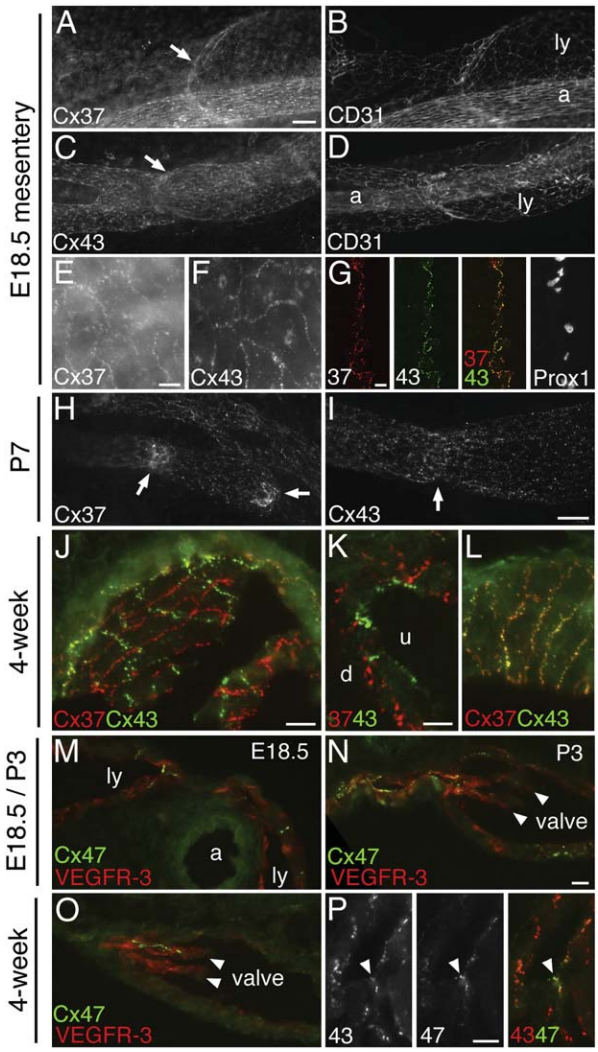


**Fig. 2.** Cx37, Cx43, and Cx47 become progressively enriched at mesenteric lymphatic valves and are differentially expressed in mesenteric valve leaflets.(A–G) Immunolabeling of mesenteric collecting lymphatics (ly) of E18.5 WT embryos. Whole-mount immunolabeling shows Cx37 and Cx43 labeling throughout the Ly endothelium, including at valves (arrows). (B and D) CD31 labeling of the same sections shown in (A and C) respectively. Arteries are labeled (a). (E and F) Higher magnification of Cx37 and Cx43 labeling shows that the Cx immunosignals outline individual endothelial cells. (G) Cx37 (red) and Cx43 (green) colocalize in this transverse section through a mesenteric Ly vessel (non-valve region). The far-right panel shows Prox1 labeling of an adjacent section. (H and I) At P7, Cx37 is enriched at mesenteric Ly valves (arrows) whereas Cx43 is more uniformly expressed in the Ly endothelium. (J and K) At postnatal 4-weeks, Cx37 (red) and Cx43 (green) are highly enriched at mesenteric Ly valves and are differentially localized in the valve leaflets. A flat, *en face* view of a valve leaflet is shown in (J) and a transverse section of a leaflet is shown in (K). Cx43 is enriched in the upstream side of the leaflet (u) and Cx37 is enriched in the downstream side (d). (L) Labeling of an oblique section through the wall of a 4-week postnatal mesenteric Ly shows that Cx37 and Cx43 colocalize in non-valve Ly endothelium. (M–P) Cx47 expression in mesenteric lymphatics at different stages of development. (M) At E18.5, Cx47 is found in a subset of LECs in the mesenteric lymphatics. (N) At P3, Cx47 is found associated with valve leaflets (arrowheads). (O) At 4 weeks, Cx47 is highly restricted to a subset of cells within valve leaflets (arrowheads). In valve LECs expressing Cx47, Cx47 and Cx43 colocalize (arrowheads in panel P). In (M–O) VEGFR-3 labeling identifies the lymphatics. Scale bars: (A–D) 50 μm; (E and F) 10 μm; (G) 10 μm; (H and I) 50 μm; (J and L) 10 μm; (K) 5 μm; (M–O) 10 μm; (P) 10 μm.

*Cx37, Cx43, and Cx47 are present in lymphatic vessels of the adult mouse and are differentially expressed in valves of the thoracic duct*

Cx expression in adult mouse Ly vessels was also examined, starting with the TD, the largest Ly vessel of the body. The TD originates with the confluence of the lumbar and intestinal Ly trunks of the abdomen and extends anteriorly through the chest and into the neck, where it delivers lymph to the venous system in the area of the left jugular and subclavian veins. We detected Cx37 (Fig. 3A) and Cx43 (Fig. 3B), but not Cx26, Cx40, Cx45, or Cx47 in the general Ly endothelium (non-valve regions) of the vessel. Levels of Cx37 were comparatively much lower in the TD than in arterial endothelium (Fig. 3C). Cx43 expression in the TD was more variable than Cx37, and detection required a high affinity antibody. When both Cx37 and Cx43 were detected in the same sections of TD, the staining colocalized (Fig. 3D) (except in valves, as discussed below).

Cx37, Cx43, and Cx47 were found to be strikingly enriched in the valves of the TD (Fig. 3E, F, and I). As in mesenteric Ly valves, Cx37 and Cx43 displayed remarkably distinct expression patterns in TD valves (Fig. 3G and H). Since valve leaflets are comprised of two endothelial layers separated by a thin extracellular matrix (Takada, 1971) (see Fig. S1 in the supplemental material for electron microscopy), we hypothesized that Cx37 and Cx43 might be differentially expressed in the two endothelial layers. To address this possibility, we used confocal microscopy to examine sections of TD and mesenteric lymphatics where the valve leaflet was relatively flat and intact. Analysis of the z-stack series of these *en face* sections showed that Cx37 was highly expressed on the downstream side of the valve leaflet, whereas Cx43 was enriched on the upstream side (Fig. 3H and see also Fig. S2 in the supplemental material for schematic summary). Endothelial cells on the downstream side of the leaflet tended to be more elongated and densely spaced than cells on the upstream side. Consistent with the mesentery data, Cx47 was found exclusively in the valve leaflets of the TD (Fig. 3I), typically in a subset of LECs that tended towards the base of the valve, where it colocalized with Cx43 (Fig. 3J). We also sampled a few other Ly vessels in the adult for Cx37 and Cx43 expression, including deep lymphatics of the submaxillary and axillary regions, and confirmed expression of both Cxs (not shown). In the peripheral lymphatics of the ear, Cx37 and Cx43 were detected in collecting lymphatics (Fig. 3K and L) but not in the LYVE-1 positive Ly capillaries (Fig. 3M and N).

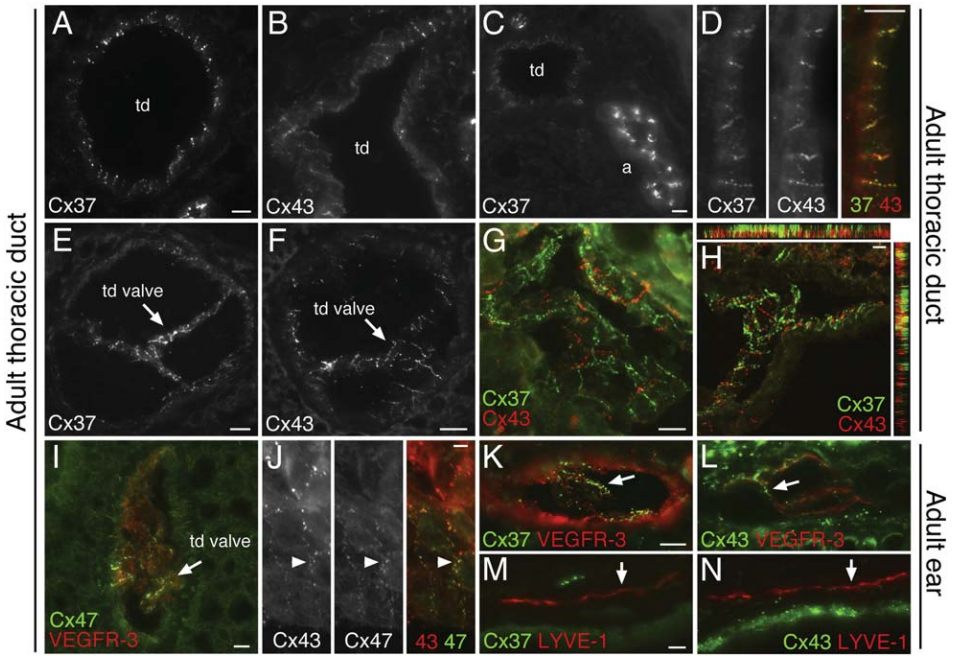


**Fig. 3.** Cx37, Cx43, and Cx47 are highly enriched in lymphatic valves in the adult mouse and are differentially expressed in upstream and downstream sides of thoracic duct valves. (A–H) Immunolabeling of thoracic duct (td) transverse sections from adult WT mice for Cx37 and Cx43. Cx37 and Cx43 are both detected in the endothelium of the TD general wall (non-valve region) and colocalize there (D). (C) Cx37 expression is comparatively much lower in TD than in arteries (a). (E and F) Cx37 and Cx43 are highly enriched in valves of the TD. (G) Epifluorescent imaging of a flat, *en face* TD valve section colabeled for Cx37 (green) and Cx43 (red) shows that the two Cxs are differentially localized. (H) Confocal z-stack imaging of another flat, *en face* TD valve section colabeled for Cx37 and Cx43 shows that Cx43 is enriched in the upstream side of the leaflet and Cx37 is enriched in the downstream side. In the center is the x–y projection through the z-stack. At the top is the x–z projection and at the right is the y–z projection, both showing separation of the Cx37 and Cx43 signals. (I and J) Cx47 is found exclusively in a subset of cells of the valve in the TD (a flat, *en face* portion of the valve is shown), where it colocalizes with Cx43 (arrowheads in J). (K–N) Immunolabeling of Cx37 and Cx43 in the ear of adult mice. Cx37 and Cx43 (both green) are detected in valved collecting lymphatics of the ear (arrows in K and L) but not in the Ly capillaries highly expressing LYVE-1 (red) (arrows in M and N). In (I, K, and L) VEGFR-3 labeling highlights the lymphatics. Scale bars: (A and B) 10 μm; (C) 10 μm; (D) 10 μm; (E and F) 20 μm; (G–I) 10 μm; (J) 5 μm; (K and L) 10 μm; (M and N) 10 μm.

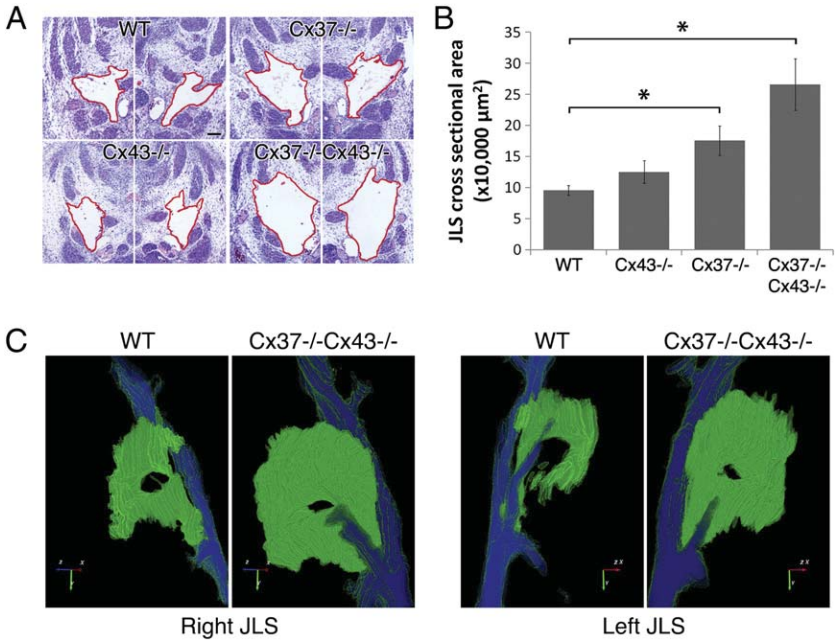


**Fig. 4.** Cx37−/− and Cx37−/−Cx43−/− embryos have enlarged jugular lymph sacs at E13.5. (A) H&E stained transverse sections of E13.5 mouse embryos. Sections were taken just above the point where the cervical nerve crosses the JLS. The paired right and left JLS lumen is outlined in red for each genotype. (B) Cross-sectional area measures of the JLS for the various genotypes show that the JLS is significantly enlarged in Cx37−/− and Cx37−/−Cx43−/− embryos compared to WT (asterisk indicates $p<0.05$). Error bars indicate s.e.m. (C) 3D volume reconstruction of JLS (green) and cardinal vein (blue) from serially sectioned WT and Cx37−/−Cx43−/− E13.5 embryos. The volume of the JLS in the Cx37−/−Cx43−/− embryo was much larger (~6-fold) than in the WT control. Axes: z (blue), dorsal; y (green), caudal; and x (red), lateral. Scale bar: (A) 100 μm.

*Cx37−/− embryos have enlarged jugular lymph sacs at E13.5*

We next examined Cx37−/− and Cx43−/− mice, as well as mice deficient in both Cxs during embryogenesis to investigate the role Cx37 and Cx43 play in Ly development, starting with the JLS. Morphometric analysis revealed that the JLS cross-sectional area at E13.5 was greatly enlarged in Cx37−/− (1.7-fold) and Cx37−/− Cx43−/− (2.6-fold) embryos compared to WT embryos but was not significantly altered in Cx43−/− embryos (Fig. 4A and B). 3D volume reconstructions from serial sections showed that the total JLS volume in Cx37−/−Cx43−/− embryos (n = 2) was also much larger (~6-fold) than in a WT control (Fig. 4C).

*Cx37−/−Cx43−/− embryos display lymphedema, abnormal thoracic duct development, and blood-filled lymphatics*

At E18.5 or P0, Cx37−/−Cx43−/− mice exhibited severe edema, particularly encircling the neck (nuchal edema) (Fig. 5A). Cx37+/− Cx43−/− mice also showed edema but at a lower frequency. In contrast, edema was seldom observed in Cx37−/− (Fig. 5B) or Cx43−/− (Fig. 5C) embryos or newborns. Sections of E16.5 Cx37−/− Cx43−/− embryos revealed widely dilated superficial lymphatics in the skin as well as a brawny thickening of the subcutaneous tissue characteristic of lymphedema (Fig. 5D and F). Dilated superficial lymphatics were not observed in E16.5 Cx37−/− or Cx43−/− embryos. We also looked at TD morphology in E18.5 embryos by whole-mount Prox1 immunostaining. WT TDs (Fig. 6A) exhibited relatively uniform caliber at this stage whereas TDs of Cx43−/− (Fig. 6C) and Cx37−/−Cx43−/− (Fig. 6D) embryos displayed extremely erratic caliber as well as blind-ended outcroppings and

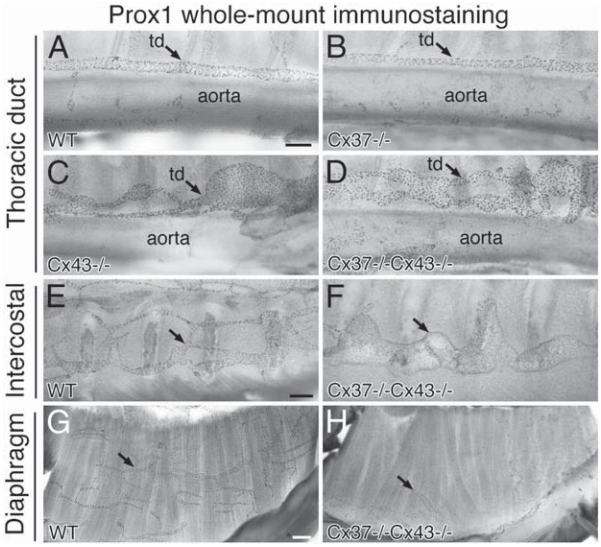


**Fig. 6.** Central lymphatic patterning is abnormal in Cx43−/− and Cx37−/−Cx43−/− embryos at E18.5. Prox1 whole-mount immunostaining of E18.5 thoracic duct (td) (A–D), E18.5 intercostal Ly trunks (E and F), and E18.5 diaphragm muscle (G and H). Cx43−/− and Cx37−/−Cx43−/− TDs have extremely erratic caliber, blind-ended outcroppings, and bifurcated segments compared with WT and Cx37−/− TDs. The intercostal trunk Lys (arrows) in a Cx37−/−Cx43−/− embryo (F) are sac-like compared to the WT control (E). The Ly network (arrows) on the thoracic surface of the diaphragm muscle is diminished in the Cx37−/−Cx43−/− embryo compared to the WT control. Scale bars: (A–D) 200 μm; (E and F) 200 μm; (G and H) 200 μm.

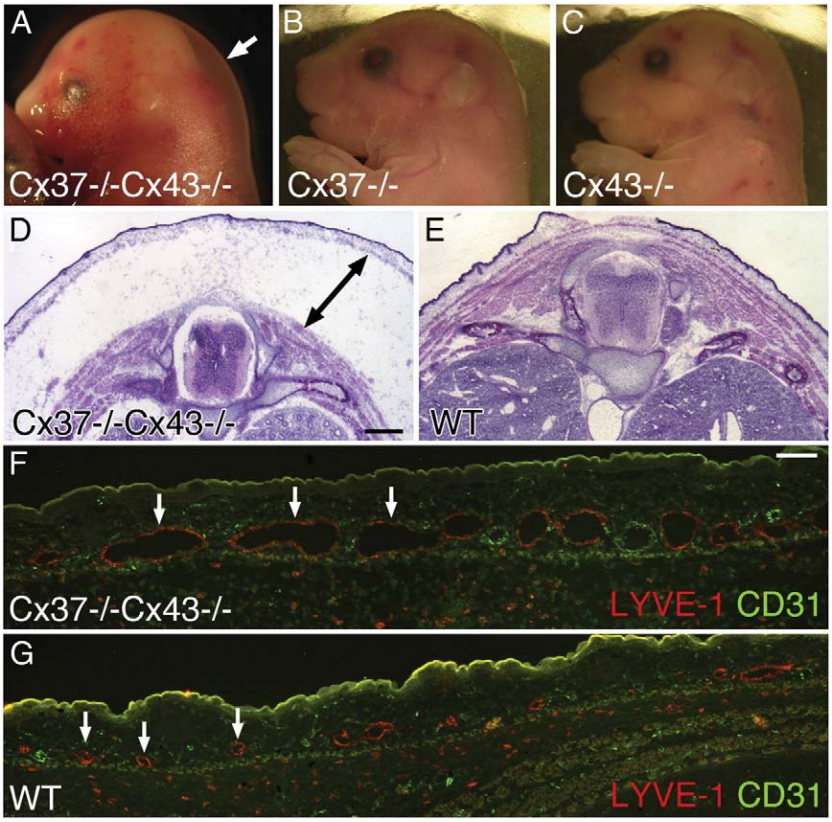


**Fig. 5.** Cx37−/−Cx43−/− embryos exhibit lymphedema and dilated superficial lymphatics. (A–C) Upper portion of embryos at E18.5. Cx37−/−Cx43−/− embryos exhibit edema whereas Cx37−/− and Cx43−/− embryos do not. (D and E) H&E stained transverse sections of E16.5 embryos show evidence of massive edema (thickening of the subcutaneous tissue is marked by a double arrow) in the Cx37−/−Cx43−/− embryo (D) but not in the WT control (E). (F and G) Transverse sections of E16.5 embryos labeled for LYVE-1 (red) and CD31 (green). The LYVE-1 labeling reveals widely dilated superficial lymphatics (arrows) in the Cx37−/−Cx43−/− embryo (F) but not in the WT control (G). Scale bars: (D and E) 400 μm; (F and G) 100 μm.

bifurcated segments. In addition, the morphology of the left and right intercostal Ly trunks was abnormal (Fig. 6F). In contrast, the TD and intercostal trunks looked normal in Cx37−/− embryos (Fig. 6B). Finally, the Ly network on the thoracic surface of the diaphragm muscle, visualized by Prox1 staining, was diminished in Cx43−/− and Cx37−/−Cx43−/− E18.5 embryos (Fig. 6H), suggesting that there was reduced invasion of Ly sprouts into this muscle.

Cx37−/−Cx43−/− embryos and Cx37+/−Cx43−/− embryos often presented with significant amounts of blood in the Ly vasculature (Fig. 7A and B). At E18.5 or P0, Cx37−/−Cx43−/− mice were usually identifiable in litters by the bloody discoloration in superficial skin lymphatics. We confirmed that the large bloody vessels in the skin were lymphatics rather than blood vessels by whole-mount Prox1 staining (Fig. 7C). Blood was also present in the TD (Fig. 7D and E) and intercostal Ly trunks (Fig. 7D and F) of E18.5 Cx37−/−Cx43−/− embryos and could be followed through Ly trunks into a central sac-like area of the mesentery and then into the mesenteric collecting lymphatics and the superficial lymphatics of the intestinal wall (Fig. 7G). Prox1 staining confirmed the Ly identity of the blood-filled vessels in the mesentery (Fig. 7H). Mesenteric blood vessels in E18.5 Cx37−/−Cx43−/−, Cx37+/−Cx43−/−, and Cx43−/− embryos looked normal, and aberrant connections between blood vessels and Ly vessels were not observed. The frequency of Ly defects for E18.5-P0 Cx-deficient mice is summarized in Table S1 in the supplemental material.

*Cx37 and Cx43 are required for lymphatic valve development in collecting vessels of the mesentery and skin*

We considered the possibility that some of the Ly disorders in Cx knockout embryos, including bloody lymphatics, might be due to an abnormality in Ly valves. The number of valves in mesenteric collecting lymphatics of WT, Cx37−/−, Cx43−/−, and Cx37−/−Cx43−/− embryos were compared at E18.5. In WT embryos (Fig. 8A, B, and I), valves were abundant in the mesenteric collecting lymphatics (26.3 ± 1.9 valves/mesentery) and could be visualized easily by whole-mount immunostaining for Prox1 and CD31. Prox1 expression was elevated in valve LECs and CD31 staining also highlighted the valve leaflets. However, Ly valves were completely absent in Cx43−/− (Fig. 8E and I) and Cx37−/−Cx43−/− (Fig. 8G and I) mesentery samples. In addition, collecting lymphatics of Cx43−/− and Cx37−/−Cx43−/− embryos often appeared jagged or enlarged, and LEC morphology was somewhat disorganized (see Fig. S5J in the supplemental material, for example). Furthermore, compared to WT, there was a 47% reduction in the number of valves in Cx37−/− mesentery (13.9 ± 1.5) (Fig. 8C, D, and I) and a 23% reduction in valves in Cx37+/−Cx43+/− mesentery (20.3 ± 1.8) (Fig. 8I). Finally, whole-mount Prox1 immunostaining of the thoracic cavity showed that valves were also absent from the intercostal Ly trunks of E18.5 Cx43−/− and E18.5 Cx37−/−Cx43−/− embryos (see Fig. S3 in the supplemental material).

Valves were also examined in collecting lymphatics of the skin from E18.5 embryos by whole-mount immunostaining (see Fig. S4 in the supplemental material). In WT embryos, valves could be detected in the dorsal skin at this stage but these were entirely absent in the Cx37−/−Cx43−/− samples, and the lymphatics were often dilated or sac-like. In contrast to the mesentery, however, some valves were still present in Cx43−/− skin samples, although the frequency of valves appeared reduced. Valves were also retained in Cx37−/− skin samples. Thus, although the loss of Cx43 alone is sufficient to completely prevent valve formation in mesenteric collecting lymphatics and intercostal lymphatics, skin lymphatics require the loss of both Cx37 and Cx43 before valves fail to form entirely.

Valves might not form in Cx-deficient lymphatics if these vessels fail to initiate the developmental program for formation of collecting lymphatics. To address this issue, we examined whole-mount mesentery collected at E17.5-P0 for expression of a number of

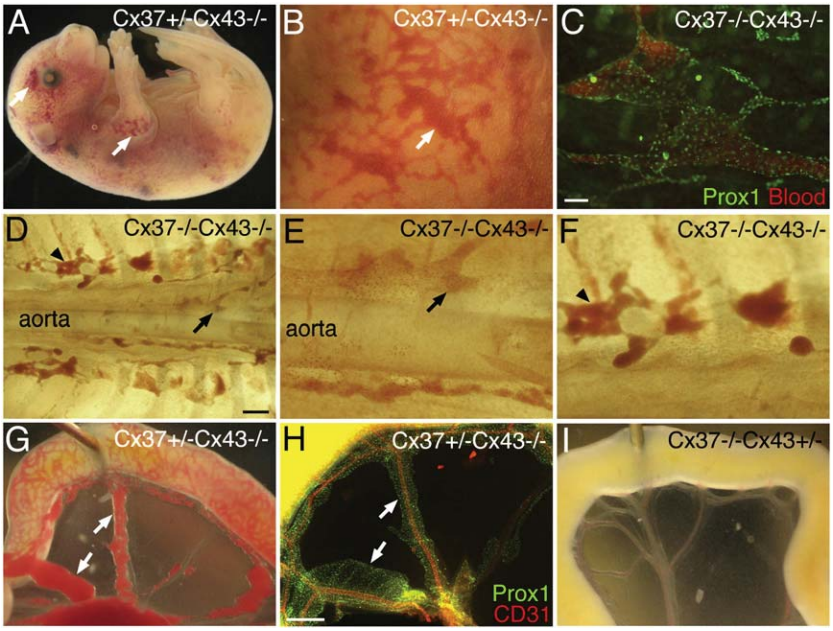


**Fig. 7.** Cx37+/−Cx43−/− and Cx37−/−Cx43−/− embryos display blood-filled lymphatics. (A) Bloody discoloration in superficial skin lymphatics of a Cx37+/−Cx43−/− E18.5 embryo. (B) Higher magnification view of blood in superficial skin lymphatics of a Cx37+/−Cx43−/− E18.5 embryo. (C) Whole-mount skin Prox1 (green) immunostaining of a Cx37−/−Cx43−/− embryo confirms the Ly identity of the bloody vessels. Blood (red) was detected by autofluorescence with UV excitation. (D) Bloody discoloration in the TD (arrow) and intercostal Ly trunks (arrowhead) of a Cx37−/−Cx43−/− E18.5 embryo. Note: the specimen was photographed after whole-mount immunostaining for Prox1 (faint spots) and blood that was in the aorta has been washed away during tissue processing. (E and F) Higher magnification views of bloody TD (arrow) and bloody intercostal Ly trunks (arrowhead) shown in (D). (G) Blood-filled mesenteric collecting lymphatics (arrows) as well as bloody superficial intestinal lymphatics are observed in this Cx37+/−Cx43−/− E18.5 specimen. (H) The same specimen as in (G) stained for Prox1 (green) and CD31 (red) confirms the Ly identity of the large blood-filled vessels (arrows point to the same vessels as in G). (I) A Cx37−/−Cx43+/− specimen shows no blood in mesenteric or intestinal lymphatics. Scale bars: (C) 100 μm; (D) 400 μm; and (H) 400 μm.

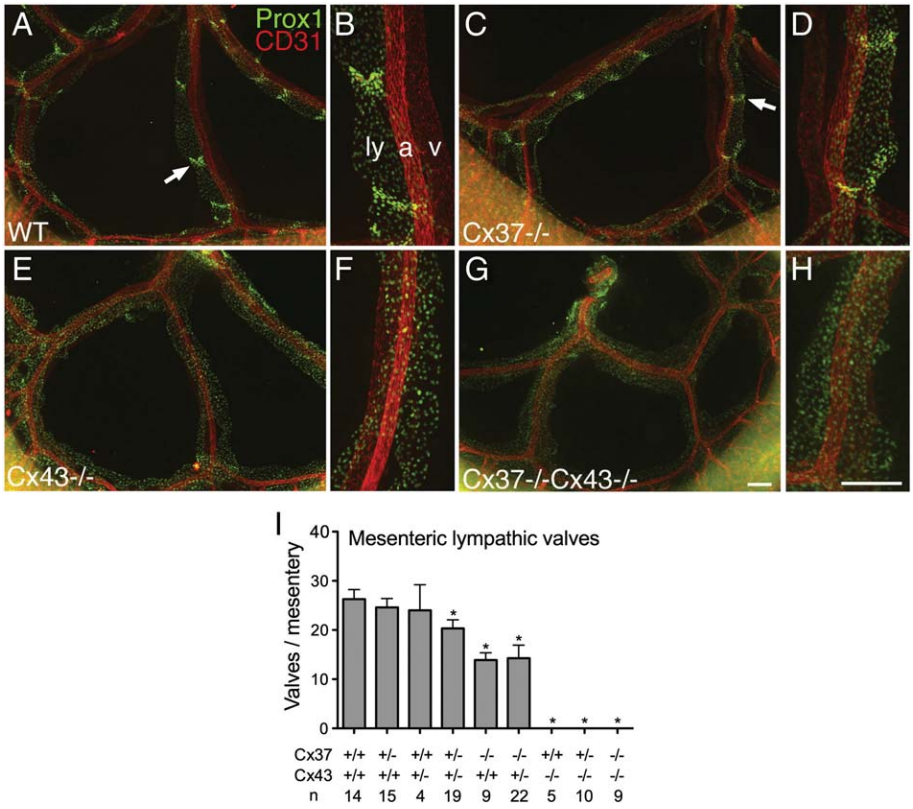


**Fig. 8.** Cx37 and Cx43 are required for lymphatic valve development in collecting vessels of the mesentery. (A–H) Whole-mount immunolabeling of E18.5 mesentery samples for Prox1 (green) and CD31 (red). The intestine is at the bottom of the field. (B, D, F, and H) are higher magnification views of (A, C, E, and G), respectively. Valves, highlighted by elevated Prox1 expression, are found in WT (A and B) and Cx37−/− (C and D) samples (arrows) but not in Cx43−/− (E and F) or Cx37−/−Cx43−/− (G and H) specimens. (I) Quantification of valves in E18.5 mesenteries showed that valves are completely absent in specimens lacking Cx43. Compared to WT, there was a 47% reduction in the number of valves in Cx37−/− mesentery. Asterisks indicated a statistically significant difference from WT (Cx37+/+Cx43+/+), $p<0.05$ for Cx37+/−Cx43+/−; $p<0.01$ for the others. Error bars indicate s.e.m. The number of mesenteries analyzed for each genotype is indicated at the bottom. ly, lymphatic. a, artery. v, vein. Scale bars: (A, C, E, and G) 200 μm; (B, D, F, and H) 200 μm.

markers whose expression has been characterized during collecting vessel development (Norrmén et al., 2009), including Foxc2, NFATc1, LYVE-1, and VEGFR-3 (see Fig. S5 in the supplemental material). In all cases, marker immunostaining in Cx43−/− or Cx37−/−Cx43−/− mesenteric lymphatics appeared similar to controls, except for the absence of valve staining. NFATc1 was detected in the nucleus in both Cx43−/− and Cx43+/− mesentery, suggesting that there was no difference in its activation state. LYVE-1 showed normal down regulation from relatively high levels at E18.5 to lower levels at E19.5 or P0, as expected during collecting vessel maturation (Norrmén et al., 2009). We also immunostained mesentery for smooth muscle actin and NG2 chondroitin sulfate as markers for mural cell recruitment. At E18.5, there was very little smooth muscle cell actin or NG2 staining of WT mesenteric lymphatics, and this feature was not altered in Cx43−/− mesentery. NG2 staining was also unchanged in Cx43−/− and Cx37−/−Cx43−/− skin lymphatics.

Ly valve defects have also been observed in mice with mutations in EphrinB2 (Mäkinen et al., 2005) or integrin-α9, and one interesting possibility is that Cx37 or Cx43 might interact with EphrinB2 or integrin-α9 in Ly vessels (Bazigou et al., 2009; Huang et al., 2000). As an initial step towards investigating this scenario, we compared the distribution of Cx37 and Cx43 with that of EphrinB2 and integrin-α9 in WT Ly vessels and looked for colocalization (see Fig. S6 in the supplemental material). In whole-mount P4 mesentery, Cx37 and integrin-α9 partially overlapped in the valve leaflets where both were substantially concentrated. Cx37 was often more highly expressed towards the base of the valve. In addition, Cx43 and EphrinB2 colocalized in distinct punctae in some submucosal lymphatics of the E18.5 intestine.

### *Cx37−/−Cx43+/− mice display retrograde lymph flow and chylothorax*

We next turned our attention to Cx37−/−Cx43+/− mice, which show reduced postnatal viability. Only ~50% of the expected frequency of this genotype was obtained at weaning (see Table S2 and Table S3 in the supplemental material). Moreover, many of the Cx37−/−Cx43+/− mice that survived to weaning died suddenly and prematurely in the following weeks, with ~40% of the deaths occurring before 8 weeks (Fig. 9A). Necropsy revealed a milky effusion around the heart and lungs consistent with chylothorax, a disorder resulting from a leak or disruption of the TD or one of its chyle (intestinal lymph) containing tributaries (Fig. 9B). We measured triglycerides, cholesterol, protein, and the cellularity of the fluid and the results confirmed its chylous nature. In particular, it was high in triglycerides (2740 ± 845 mg/dL; n = 5) and cholesterol (196 ± 47 mg/dL; n = 5) and the cholesterol/triglycerides ratio (0.11 ± 0.03; n = 5) was typical of chyle. Many of the cells (~70%) present in the fluid were lymphocytes. Chylothorax was not observed in Cx43+/− mice and was extremely rare in Cx37−/− mice (one case out of ~300 mice).

To assess Ly function in Cx37−/−Cx43+/− mice, we performed Evans blue dye (EBD) visual lymphangiography to follow lymph drainage patterns. When EBD is injected into the dermis of the hindpaws, the dye is taken up by Ly capillaries, binds tightly to protein in tissue fluid and lymph, and is transported through the Ly trunks of the leg and abdomen, through the iliac and retroperitoneal lymph nodes, and eventually into the TD. EBD can therefore normally be traced from the injected hindpaw all the way through the TD. With WT mice, we observed the expected unidirectional anterograde transport of EBD, and in the chest wall, the dye was restricted to the TD as it moved cephalad (Fig. 9C). In contrast,

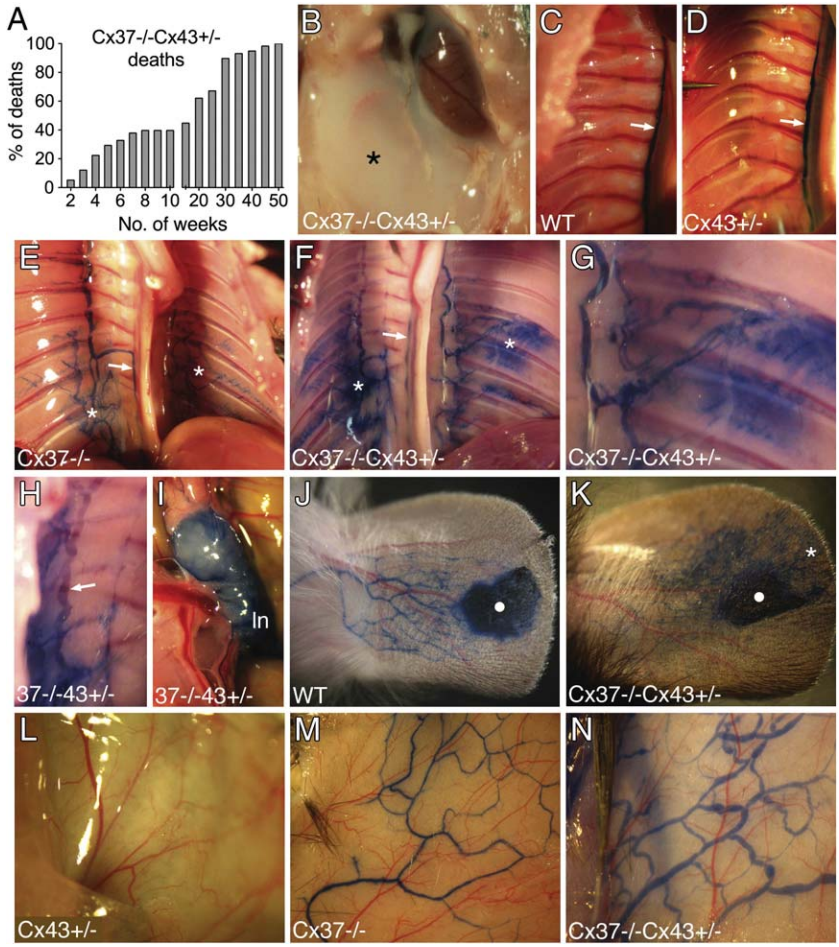


**Fig. 9.** Cx37−/−Cx43+/− mice display retrograde lymph flow and frequently die prematurely with chylothorax. (A) Percentage of deaths of Cx37−/−Cx43+/− mice versus age. Approximately 40% of the deaths occurred before 8 weeks of age. (B) Milky chylous effusion surrounding the heart and lungs of a Cx37−/−Cx43+/− mouse with chylothorax. (C–N) EBD visual lymphangiography was performed to follow lymph drainage patterns. (C and D) Following hindpaw injection of EBD, in WT and Cx43+/− mice the dye is restricted to the TD (arrow) as it moves unidirectionally cephalad. (E–G) In many Cx37−/− and Cx37−/−Cx43+/− mice, EBD shows reflux (retrograde flow) into intercostal lymphatics (asterisks) lateral to the TD (arrow). (G) A higher magnification view of area of intercostal dye reflux marked by asterisk in (F). (H) In some Cx37−/−Cx43+/− mice, blood is observed in the same intercostal Ly vessels that contains EBD due to reflux (arrow). (I) Cx37−/−Cx43+/− mice show a high incidence of EBD reflux into mesenteric lymph nodes (ln). (J) EBD injection into the dermis of the ear of a WT mouse (injection site is marked by a white circle) results in unidirectional drainage and convergence of the dye into Ly collecting vessels. (K) Reflux (marked by an asterisk) and increased lateral spread occurs when EBD is injected into the ear of a Cx37−/−Cx43+/− mouse. (L–N) Hindlimb skin in the area above the EBD-injected hindpaw. The Cx43+/− hindlimb skin (L) shows no dye reflux whereas hindlimb skin from Cx37−/− (M) and Cx37−/−Cx43+/− (N) mice show prominent reflux of EBD into a network of surrounding lymphatics in the skin.

with Cx37−/−Cx43+/− mice, we noted reflux (retrograde flow) of EBD into a network of surrounding lymphatics in the hindlimb skin (Fig. 9N), as well as reflux into mesenteric lymph nodes (Fig. 9I) and intercostal lymphatics (Fig. 9F and G). Reflux and increased lateral spread of EBD also occurred when the dye was injected into the dermis of the ear (Fig. 9K). While all the Cx37−/−Cx43+/− mice exhibited EBD reflux into the hindlimb skin lymphatics, intercostal dye reflux varied from severe to mild or, less commonly, was undetectable. When EBD was injected into the hindpaw of a mouse with ongoing chylothorax, the dye leaked into the thoracic cavity and mixed with the chylous effusion, consistent with rupture of the TD or one of its tributaries. In some Cx37−/−Cx43+/− mice, EBD did not fill the TD following hindpaw injection, indicating a severe impairment of lymph transport. Peripheral edema was not observed in Cx37−/−Cx43+/− mice, however, suggesting that the primary defect in mice with this genotype involves central rather than peripheral lymphatics. To investigate this issue further, we looked at peripheral Ly networks in the ear by whole-mount LYVE-1 immunostaining, which labels Ly capillaries. Ly patterning looked normal in ears of Cx37−/−Cx43+/− and Cx37−/− mice, and the number of branch points in the Cx37−/−Cx43+/− Ly network was not different from WT (see Fig. S7 in the supplemental material).

EBD injections were also performed with Cx37−/−, Cx43+/−, Cx37+/−Cx43+/− and Cx40−/− mice. Surprisingly, we found that Cx37−/− mice also frequently exhibited dye reflux into hindlimb skin (Fig. 9M) and intercostal lymphatics (Fig. 9E). Intercostal reflux, however, was not usually as severe in Cx37−/− mice as in Cx37−/−Cx43+/− mice, and there were also fewer instances in which the TD did not fill at all with dye. Moreover, reflux into mesenteric lymph nodes was observed in Cx37−/−Cx43+/− mice but not in Cx37−/− mice. These results indicate that although chylothorax is very rare in Cx37−/− mice, Ly function is nevertheless significantly impaired in these mice. EBD transport was normal in Cx43+/− (Fig. 9D) and Cx40−/− mice, and reflux occurred only very rarely in Cx37+/−Cx43+/− mice. The frequency of Ly defects for adult Cx-deficient mice is summarized in Table S4 in the supplemental material.

### *Cx37 and Cx43 are required for thoracic duct valve development*

The phenotype of Cx37−/−Cx43+/− mice and Cx37−/− mice suggested either a defect in the ability of truncal lymphatics to move lymph effectively in an anterograde fashion or, alternatively, an obstruction in the truncal lymphatics. Given the valve data from Cx-

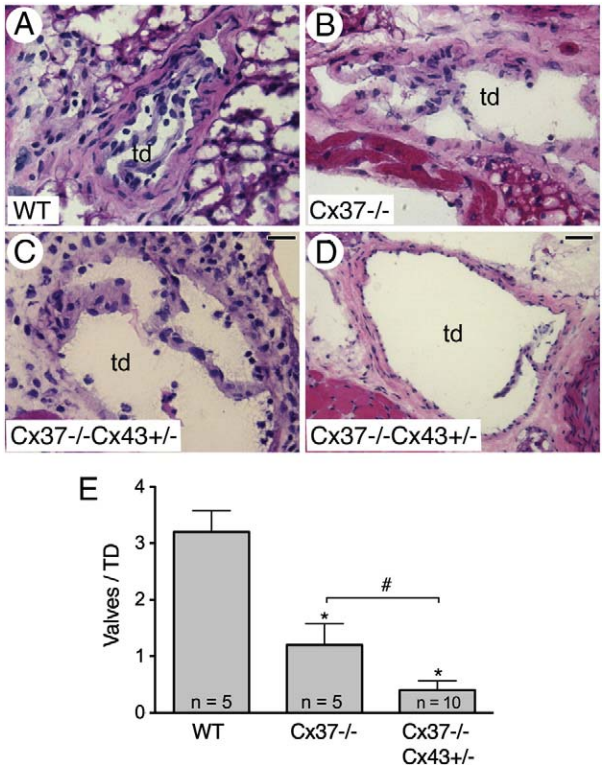


**Fig. 10.** Cx37 and Cx43 are required for thoracic duct valve development. (A–D) H&E stained transverse sections of TD (td) from adult mice. (A) Representative example of a TD valve from WT mouse, showing typical bicuspid morphology. (B) TD valves are observed in Cx37−/− mice but the frequency of valves is reduced. (C and D) Rare examples of TD valves present in Cx37−/−Cx43+/− mice. In most Cx37−/−Cx43+/− mice, however, no TD valves were found. Based on its appearance in serial sections, the valve shown in (D) was likely functionally insufficient. (E) Quantification of the number of valves per TD for WT, Cx37−/−, and Cx37−/−Cx43+/− mice. Asterisks indicate statistically significant differences from WT ($p<0.01$). There was also a statistically significant difference (denoted by #) between Cx37−/− and Cx37−/−Cx43+/− mice ($p<0.05$). Error bars indicate s.e.m. The number of TDs analyzed for each genotype is indicated within the bar. Scale bars: (A–C) 20 μm; (D) 40 μm.

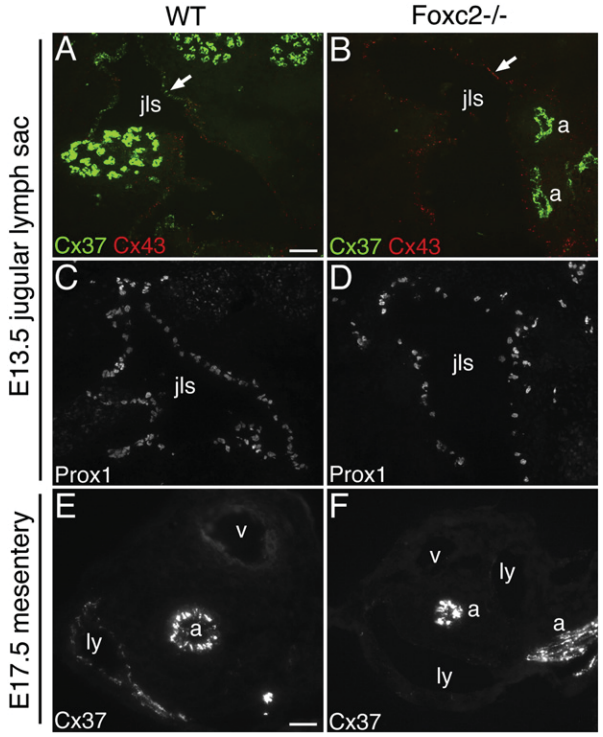


**Fig. 11.** Cx37 expression in jugular lymph sac and mesenteric collecting lymphatics is reduced in the absence of the transcription factor Foxc2. (A–D) Immunolabeling of JLS (jls) of E13.5 WT and Foxc2−/− embryos. (A) Cx37 (green) and Cx43 (red) expression in the JLS (arrow) of a E13.5 WT embryo. The Cx37 antibody crossreacts with some muscle fiber types (upper right and middle left). (B) In Foxc2−/− embryos, Cx37 expression is greatly reduced in the JLS whereas Cx43 is still present. Cx37 expression in arteries (a) is unaffected by the absence of Foxc2. (C and D) Prox1 labeling of sections adjacent to those in (A and B) respectively. (E and F) Cx37 immunolabeling of transverse sections of mesentery from E17.5 WT and Foxc2−/− embryos. (E) In WT mesentery, Cx37 is expressed by collecting lymphatics (ly) and by arteries (a). (F) Foxc2−/− mesentery shows reduced expression of Cx37 in the lymphatics but not in the arteries. Scale bars: (A–D) 50 μm; (E and F) 20 μm.

deficient embryos, we hypothesized that Cxs are also necessary for normal development of the TD valves. To test this idea, an extensive histological analysis of excised TDs from adult Cx37−/−Cx43+/−, Cx37−/−, and WT mice was performed (TDs from Cx43−/− and Cx37−/−Cx43−/− mice could not be examined as those genotypes are not viable postnatally). TDs were serially sectioned (transversely) from just above the diaphragm all the way to the top of the heart, and sections were either stained with H&E or immunostained with Prox1 to identity the TD and to visualize valves. In WT mice, valves (typically bicuspid) were present only in the rostral half of the TD and were more closely spaced towards the top (Fig. 10A and see Fig. S8 in the supplemental material for a schematic of valve distribution). We observed up to four valves (3.20±0.37 valves/TD) in the WT TDs (Fig. 10E). In contrast, the number of valves in TDs from Cx37−/−Cx43+/− mice was reduced by ~10-fold (0.30±0.15 valves/TD) (Fig. 10E). In most of the Cx37−/−Cx43+/− mice, no TD valves were found while others had only one valve. Cx37−/− mice also had a substantial reduction in TD valves (1.20±0.37 valves/TD) (Fig. 10E), but there were clearly more valves in Cx37−/− mice than in Cx37−/−Cx43+/− mice. These results support the hypothesis that Cx37 and Cx43 are required for TD valve development and provide a plausible explanation for the Ly functional defects observed in Cx37−/−Cx43+/− and Cx37−/− mice. We also considered the possibility that the TD was compromised in Cx-deficient mice by a deficiency in associated smooth muscle cells. Sections of Cx37−/−Cx43+/− and WT TD were immunostained for smooth muscle cell actin but no differences in staining were observed (see Fig. S9 in the supplemental material).

A number of additional Ly phenotypes were noted sporadically in the analysis of Cx37−/−Cx43+/− mice (see Fig. S10 in the supplemental material). These included blood in the lumen of the TD, large aggregates of immune cells obstructing the duct, atresia of the duct, and wide dilation of the duct. The immune cell aggregates were CD45+ leukocytes and contained a mixture of F4/80+ macrophages/monocytes, CD3+ T cells, and CD19+ B cells. In addition, we observed cases of abnormal sharp turns in the TD and instances in which the duct was on the opposite side of the midline. Anatomical aberrations of this type were also noted with some frequency in Cx37−/− mice. In one instance, a Cx37−/− mouse was found to have a grossly overgrown and convoluted TD valve. Finally, significant abnormal fat deposition in the thoracic cavity was noted in some Cx37−/−Cx43+/− mice, particularly along the aorta and TD and around the heart.

### *Cx37 expression in jugular lymph sac and mesenteric collecting lymphatics is reduced in the absence of the transcription factor Foxc2*

Because Foxc2 has been implicated in Ly valvulogenesis (Kriederman et al., 2003; Norrmén et al., 2009; Petrova et al., 2004), we hypothesized that Cx37 and Cx43 might be downstream of this forkhead family transcription factor in the collecting vessel developmental pathway. To test this idea, we compared Cx37 and Cx43 expression in WT and Foxc2−/− embryos, which fail to initiate the collecting vessel

developmental program (Norrmén et al., 2009). Foxc2−/− embryos die variably between E12.5 and P0, so embryos were initially collected at E13.5, and Cx expression was examined in the JLS. Cx43 expression was unaffected by the loss of Foxc2 (Fig. 11B). Cx37 expression, however, was dramatically reduced in the JLS of Foxc2−/− embryos (Fig. 11B). In a few sections, residual expression of Cx37 was observed. In most sections, however, Cx37 was undetectable in the Foxc2−/− JLS whereas Cx37 expression in nearby arteries was unaffected. Interestingly, similar to Cx37−/− embryos, we noted that the JLS of Foxc2−/− embryos were greatly enlarged compared to WT controls. We also examined Cx37 expression in E17.5 mesenteric lymphatics and found that Cx37 expression was reduced but not eliminated in the Foxc2−/− embryos (Fig. 11F) compared to WT embryos (Fig. 11E).

## Discussion

In this study, we report for the first time that the GJ proteins Cx37, Cx43, and Cx47 are expressed in developing and mature Ly vessels of the mouse and that Cx37 and Cx43 are necessary for the formation of valves in Ly collecting vessels. Mice deficient in these Cxs either lack valves entirely in collecting vessels or have reduced numbers of valves, depending on the genotype. Doubly deficient Cx37−/− Cx43−/− mice also develop severe lymphedema and exhibit bloody lymphatics. Additionally, we find that Cx43 is required for normal patterning of the TD and that both Cx37 and Cx43 are important for TD valve development. Cx37−/−Cx43+/− mice are initially viable but are severely deficient in TD valves and develop lethal chylothorax. Furthermore, we show that another Cx family member, Cx47, is highly enriched in valve leaflets postnatally. Finally, we demonstrate that Cx37 expression in developing lymphatics depends, at least in part, upon Foxc2 expression, suggesting that Cx37 may be a target of Foxc2 regulation during Ly development. These data indicate that multiple Cxs are essential for normal development and function of the Ly vasculature.

Considering the marker expression data, Cx37 and Cx43 seem to be required specifically for Ly valve development rather than more generally required for collecting vessel development. In comparison, other mouse models like Foxc2−/− (Norrmén et al., 2009) and Ang2−/− (Dellinger et al., 2008; Gale et al., 2002) mice fail to initiate the collecting vessel developmental program. Thus, Cx37 and Cx43 appear to be downstream factors in the pathway and may play a direct role in coordinating LECs during valvulogenesis. The signals that locally instruct LECs to proliferate, migrate, and organize into valve leaflets are not known. Since Cx37 and Cx43 are initially expressed throughout the Ly endothelium of the developing collecting lymphatics, they could function to locally communicate second messengers at sites where a signal for valve formation is received or initiated by the Ly endothelium. A mechanism of this sort has been suggested to occur during atrioventricular valve development in the heart, where expression of Cx45 in the cardiac endothelium is required for normal endocardial cushion formation (Kumai et al., 2000; Nishii et al., 2001). In the absence of Cx45, signaling through NFATc1 was blocked in the cardiac endothelium, and a critical epithelial-mesenchymal transformation of the endothelium was impaired. Another feature of Foxc2−/− mice and Ang2−/− mice is that both models exhibit premature recruitment of smooth muscle cells to developing lymphatics (Dellinger et al., 2008; Petrova et al., 2004). It has been proposed that early association of smooth muscle cells might prematurely stabilize immature lymphatics and lead to impaired valvulogenesis (Petrova et al., 2004). In the case of Cx-deficient mice, however, we did not observe premature recruitment of smooth muscle cells to lymphatics. Thus, abnormal association of mural cells during Ly development is not a prerequisite event for valve agenesis to occur.

Since Cx43−/− mice die perinatally with cardiac developmental defects (Reaume et al., 1995), LEC-specific ablation of Cx43 will ultimately be required to determine if the cardiovascular anomalies in Cx43−/− mice contribute at all to the Ly defects in those mice. Ly defects also occur in Cx37−/− and Cx37−/−Cx43+/− mice, however, and these genotypes do not exhibit the cardiac defects associated with Cx43−/− mice, arguing that Cxs do have LEC-specific roles consistent with their expression in Ly vessels. Moreover, Ly defects were not reported for mice with a cardiac-restricted knockout of Cx43 (Gutstein et al., 2001) nor were they reported for mice with a conditional knockout of Cx43 from thoracic neural tube and cardiac neural crest, a model which reproduces the cardiac defects (infundibular bulging and abnormal coronary vascular development) seen in global Cx43 knockout mice (Liu et al., 2006).

Although lymph–blood mixing can occur if there is a failure of separation between Ly vessels and blood vessels (Abtahian et al., 2003; Bäckhed et al., 2007; Bertozzi et al., 2010; Sebzda et al., 2006; Uhrin et al., 2010), we saw no evidence of inappropriate connections between Ly vessels and blood vessels of Cx37−/−Cx43−/− or Cx43−/− mice at E18.5. The bloody lymphatics in the intestinal wall and mesentery of Cx-deficient mice did not resemble the tortuous bloody vessels observed in mouse models where a nonseparation phenotype has been described (Abtahian et al., 2003). In addition, blood vessels of the mesentery looked normal in the Cx deficient mice, and no aberrant connections between blood vessels and Ly vessels within the mesentery were observed. Moreover, tissue hemorrhage was not a source of red blood cells in the lymphatics. Rather, we think that valve deficiencies in the Cx-deficient mice allow venous blood to inappropriately, and perhaps intermittently, based on differential pressures, enter the Ly vasculature. Consistent with this idea, blood was observed in the TD of Cx-deficient embryos and could be traced into the mesentery. Moreover, even in adult Cx37−/−Cx43+/− mice, blood was sometimes found within the TD.

In adult animals, Ly collecting vessels undergo spontaneous contractions which are propagated along the vessel, and it has been suggested that GJs in the Ly wall could provide a pathway for conduction of contractile activity, as in arterioles (McHale and Meharg, 1992; Zawieja et al., 1993). Since the Ly developmental defects in Cx-deficient mice occur before the recruitment of mural cells, however, the defects cannot be entirely a consequence of an inability to actively pump lymph. Nevertheless, the question of whether or not functional GJs are actually present between LECs in embryos or adult animals deserves more attention. It will be important to investigate this issue in intact vessels by electron microscopy to look for morphologically identifiable GJs and to use dye transfer assays and electrophysiological methods to determine if LECs are functionally coupled by intercellular channels. If technical issues associated with using mouse lymphatics can be overcome, it will also be essential to directly test whether propagation of Ly contraction is altered in Cx-deficient mice. Finally, given the restricted distribution of Cxs in mature collecting lymphatics, it is tempting to speculate that Cxs could play some role in controlling pacemaker activity associated with spontaneous Ly contractions (Ohhashi et al., 2005; Zawieja, 2009).

The Cx-deficient mice in this study share some features with other mouse models displaying Ly disorders. Mutations in *Ita9* (integrin-α9) (Bazigou et al., 2009; Huang et al., 2000) and *EfnB2* (ephrinB2) (Mäkinen et al., 2005) have been shown to result in Ly collecting vessel valve deficiency and to cause chylothorax, and in humans, an Integrin-α9 missense mutation has been associated with congenital chylothorax (Ma et al., 2008). Integrin-α9−/− mice have only rudimentary valve leaflets in mesenteric collecting lymphatics and the leaflets contain a disorganized fibronectin matrix core (Bazigou et al., 2009). Integrin-α9β1 has also been linked to accelerated migration of cells in some settings (Gupta and Vlahakis, 2009) and, similarly, Cx43 is important for directed cell migration, for example, in neural crest cells (Xu et al., 2001) and during coronary blood vessel development (Rhee et al., 2009). EphrinB2$^{\Delta V}$ mice, engineered with a C-terminal PDZ interaction site deleted, exhibit blood in their Ly vessels as do Cx37−/−Cx43−/− and Cx43−/− mice (Mäkinen et al.,

2005). Because of these similarities and our preliminary colocalization studies, we speculate that Cx37 or Cx43 might interact with EphrinB2 or integrin-α9 in Ly vessels and contribute to differential cell migration, sorting, and growth control during Ly development. Along with forming GJ channels and hemichannels, Cxs can contribute to signaling via protein-protein interactions. Indeed, a different Ephrin family member, EphrinB1, has been shown previously to co-immunoprecipitate with Cx43, and interaction between the two proteins was important for normal Cx43 distribution (Davy et al., 2006). Moreover, the PDZ binding domain of EphrinB1 was required for it to interact with the phosphorylated form of Cx43 (Davy et al., 2006).

Our data suggest that the primary defect in Cx37−/−Cx43+/− mice involves central lymphatics rather than peripheral lymphatics. Patterning and function of peripheral lymphatics was normal in these mice, and peripheral edema was not observed. However, the forward movement of lymph through the central lymphatics of Cx37−/−Cx43+/− mice was compromised, and this phenomenon can be explained by a severe deficiency in TD valves. EBD lymphangiography revealed retrograde reflux of the dye into collateral lymphatics along the route of lymph transport, including reflux into intercostal lymphatics. The lack of TD valves, which normally promote unidirectional fluid movement, likely results in a backup of lymph in the central lymphatics. Reflux into collateral lymphatics then occurs to accommodate the excess lymph. Eventually, the intercostal lymphatics or the TD itself becomes overburdened and rupture occurs, resulting in chylothorax. Consistent with this model, there was a good correlation between the number of TD valves in Cx37−/− and Cx37−/−Cx43+/− mice and the degree of Ly impairment.

To our knowledge, this study is the first to quantify valves in the mouse TD and to identify genes that are required for development of TD valves. We found that TDs from WT mice typically contain 3–4 valves. By comparison, adult rats and humans have an average of 11.9 and 14.7 valves per TD, respectively (Petrenko and Kruglov, 2004). Our data are consistent with those from adult rat and human showing that TD valves are usually found in the upper portion of the duct, although human fetuses may transiently have more numerous and distributed TD valves during development (Kampmeier, 1928a; Petrenko and Kruglov, 2004). It is not known if the pathways critical for valve development in collecting lymphatics (of the mesentery, for example) are the same as for TD valve development. Kampmeier described a process of valvulogenesis in the human TD that was qualitatively distinct from valvulogenesis occurring in the periphery (Kampmeier, 1928b). At present, we do not know if abnormal JLS development at E13.5 contributes to later TD valve deficiencies in Cx37−/−Cx43+/− mice.

A number of sporadic phenotypes occurred in Cx37−/−Cx43+/− mice, including abnormal fat deposition, a characteristic feature of some Ly disorders in humans, including chronic lymphedema (Harvey, 2008). In Prox1 haploinsufficient mice, adult-onset obesity occurs associated with abnormal lymph leakage from mispatterned and ruptured Ly vessels (Harvey et al., 2005). In addition, chyle was found to have adipogenic activity when added to 3T3-L1 preadipocytes in culture (Harvey et al., 2005). Our observations are consistent with this model, as abnormal fat in Cx37−/−Cx43+/− animals accumulated only in the thoracic cavity, where leakage of chyle occurred.

Differential expression of Cx37 and Cx43 occurs in the JLS at E13.5, where there is an inverse relationship between Cx37 and Cx43 expression. Cx37 and Cx43 were previously shown to have a reciprocal relationship in specific regions of the rat aorta, such as at the aortic bifurcation (Gabriels and Paul, 1998). To our knowledge, however, the present data are a unique example of such distinct heterogeneity in gene expression amongst JLS LECs. It will be important to determine what role Cxs play in the proliferation, sprouting, and remodeling that occurs in the JLS during this period. In the absence of Cx37, the JLS is clearly enlarged, suggesting that Cx37 may play an important role in regulating the proliferation of LECs of the JLS. Consistent with this model, Cx37 has been shown to have growth suppressive properties in some settings (Burt et al., 2008; Morel et al., 2010). Quantitative differences in the relative expression levels of Cx37 and Cx43 might also explain why, in skin lymphatics, the loss of both Cx37 and Cx43 is required before valves are entirely absent whereas, in mesenteric lymphatics, only the loss of Cx43 is required.

Cx37 and Cx43 were also found to be exquisitely differentially expressed in the upstream versus downstream endothelial layers of mature Ly valve leaflets which are separated in places by less than 200 nm. In the rat heart, 70- to 200-fold greater expression of Cx43 was found in the upstream versus downstream surfaces of cardiac valves, consistent with our finding of Cx43 on the upstream side of Ly valves (Inai et al., 2004). Differential expression of endothelial Cxs has also been observed in blood vessels at ostia and flow dividers (Gabriels and Paul, 1998). In these settings, shear stress and disturbed flow were implicated in the differential expression of Cxs. The upstream and downstream surfaces of Ly valves are also likely to experience distinct flow conditions and shear stress, and the differential expression of Cx37 and Cx43 in Ly valves may therefore be a response to unequal exposure to mechanical stress. Interestingly, morphological and biochemical differences between upstream and downstream sides of Ly valve leaflets have been previously noted (Bannykh et al., 1995; Ji and Kato, 2001). Thus, Cx37 and Cx43 may have adaptive physiological functions in mature lymphatics that are distinct from their developmental roles. Alternatively, we speculate that differential Cx expression in developing collecting lymphatics may play a role in establishing functional boundaries between lymphangions, the functional units of a lymph vessel that lie between contiguous valves, much like Cx43 expression and localization regulates joint location in zebrafish fins (Sims et al., 2009).

Our data on Cx expression in Foxc2−/− mice raise the possibility that the Foxc2 transcription factor directly regulates Cx37 gene (*Gja4*) expression. Interestingly, ablation of a different forkhead box gene, *Foxo1*, resulted in a large decrease (90%) in Cx37 mRNA in the E9.5 yolk sac vasculature (Furuyama et al., 2004). Moreover, another family member, Foxo3a, is implicated in the regulation of Cx37 and Cx43 expression in mouse oocytes (Liu et al., 2007). Other observations also suggest a relationship between Foxc2 and Cx37 in the Ly system. First, the JLS is enlarged in both Foxc2−/− and Cx37−/− embryos. Second, Cx37 expression is elevated in valves in postnatal mesenteric lymphatics, as is Foxc2 expression (Petrova et al., 2004). Norrmén et al. (2009) used a Chip-chip assay to compile a human genome-wide map of Foxc2 binding sites in LECs and pulled out two Foxc2 sites where the nearest gene was (*GJA1*) Cx43, although these sites were rather distant (~380 kb and ~128 kb away) from the *GJA1* locus. We directly searched for potential binding sites for Foxc2 and NFATc1 in the flanking regions of the mouse Cx37 gene (*Gja4*) using a position frequency matrix for each transcription factor consensus binding site (Norrmén et al., 2009). Candidate sites which closely match the position frequency matrix for Foxc2 and NFATc1 binding were identified within an 11 Kb region immediately downstream of the mouse Cx37 gene (*Gja4*). Future studies will aim to determine if these potential sites are functional binding sites for Foxc2 and NFATc1 and if they are used to regulate expression of Cx37 in LECs.

Recently, missense mutations in *GJC2*, encoding Cx47, were found to cause dominantly inherited lymphedema in humans (Ferrell et al., 2010). Cx47 has previously been documented in oligodendrocytes of the central nervous system but its expression in Ly vessels has not been studied (Menichella et al., 2003; Odermatt et al., 2003). We report here that Cx47 is expressed in the valves of the TD, as well as in valves of mesenteric collecting lymphatics, in a subset of LECs. We also detected Cx47 expression in embryonic Ly vessels, although it was earlier absent from the E13.5 JLS. Some of the identified human Cx47

mutations are predicted to be dominant-negative, and therefore, Cx37 or Cx43 function could be affected in LECs coexpressing these Cxs (Ferrell et al., 2010).

In conclusion, the results of this study, along with the Cx47 human lymphedema mutations described by Ferrell et al. (2010), demonstrate that at least three Cxs have critical roles in Ly development and function. Our data show that Cx37 and Cx43 participate in lymphatic valve development as well as morphogenesis of the JLS and TD. Furthermore, *Gja4* (Cx37) and *Gja1* (Cx43) are potential candidate genes for congenital chylothorax, lymphedema, and other Ly disorders caused by valve defects as well as potential targets for development of novel molecular therapeutics.

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## Supplemental Material (Figures legends S1-S10):

**Figure S1. Ultrastructure of a TD valve from an adult WT mouse shows that the valve is made up of two endothelial cell layers.**

(A-C) Transmission electron micrographs of a TD valve from a WT mouse. The valve leaflet is comprised of two endothelial layers (e, arrows) separated by an extracellular matrix (m). Fibroblasts (f) are sometime observed in the space between the two endothelial layers. The white boxed area in (A) is shown at higher magnification in (B). The white boxed area in (B) is shown at higher magnification in (C). Scale bars: (A) 5 μm; (B) 1 μm; (C) 200 nm. Methods: TD specimens were fixed in 0.1 M cacodylate buffer containing 2.5% glutaraldehyde, 2% formaldehyde, and 0.2% tannic acid. After washing, the tissue was microwave-fixed under vacuum with 1% osmium tetroxide in cacodylate buffer. After washing, the tissue was block-stained with 4% uranyl acetate and then embedded in Spurr's resin. Transmission electron microscopy was done with a Philips CM-12 electron microscope.

**Figure S2. Summary of Cx37, Cx43, and Cx47 expression in mature valved lymphatic vessels.**

The schematic diagram is based on data obtained from TD and mesenteric collecting lymphatics. For clarity, the Ly vessel in the schematic diagram is shown as a longitudinal section running along the length of the vessel. Note that the actual immunostained sections showing differential Cx expression on the upstream and downstream side of valves in Fig. 2J-L and Fig. 3 G,H are transverse sections of Ly vessels not longitudinal sections.

**Figure S3. Cx43 is required for valve development in intercostal lymphatic trunks.**

(A-D) Whole-mount Prox1 immunostaining of intercostal Ly trunks of E18.5 embryos. At this stage of development, valves (arrows) are sometimes observed in WT (A) and Cx37-/- (B) intercostal Ly trunks.

In contrast, valves are not found in Cx43-/- or Cx37-/-Cx43-/- intercostal Ly trunks. Scale bar: (A-D) 100 μm.

**Figure S4. Cx37-/-Cx43-/- E18.5 embryos lack valves in collecting lymphatics of the skin.**

(A-H) Whole-mount immunolabeling of dorsal skin from E18.5 embryos for Prox1 (green) and CD31 (red). (A,B) Numerous valves (arrow) are present in WT skin and persist in Cx37-/- skin (C,D). (E, F) Valves are observed in Cx43-/- skin, but their frequency appears to be reduced compared to WT controls. (G, H) Valves are absent from Cx37-/-Cx43-/- skin. (A, B, G, H) Confocal imaging, x-y projection of a z-stack. (C-F) Conventional epifluorescence microscopy. (A,C,E,G) 100 μm; (B,D,F,H) 50 μm.

**Figure S5. Cx-deficient mesenteric lymphatics express appropriate markers of collecting vessel development.**

(A-P) Whole-mount immunolabeling of mesentery from E18.5 embryos or P0 pups. Samples are E18.5 unless otherwise indicated. A number of markers were examined: (A,B) Foxc2; (C,D) NFATc1; (E,F,O,P) Prox 1; (G,H,M,N) VEGFR-3; (I-L) LYVE-1; (M,N) NG2; (O, P) Smooth muscle actin (SMA). Cx-deficient specimens (which lack valves due to the absence of Cx43) were compared to WT or Cx43+/- controls (which have valves, marked by arrows). For brevity, the second and fourth columns are labeled as Cx-deficient*. These samples were chosen because they lacked Cx43, however, their Cx37 genotype varied: (F,D,L) Cx43-/-; (H,J,P) Cx37+/-Cx43-/-; (B,N) Cx37-/-Cx43-/-. The controls (first and third columns) had the following genotypes: (A,I,M,G,K,O) WT; (E,C) Cx43+/-. In all cases, marker immunostaining in Cx-deficient mesenteric lymphatics appears similar to controls, except for the absence of valve staining. LYVE-1 shows normal down regulation from relatively high levels at E18.5 (J) to lower levels at P0 (L), as expected during collecting vessel maturation and similar to WT controls (I,K). In (K,L) the weakly stained lymphatic (ly) is marked by a white outline. Note that in (K,L) scattered macrophages are labeled for LYVE-1 (isolated cells stained green). Very little SMA or NG2 staining is observed in either the Cx-deficient or control mesenteric lymphatics at this E18.5 stage. Arteries (a);

Veins (v). Scale bars: (A-F) 50 μm; (G, H) 50 μm; (I, J) 50 μm; (K,L) 50 μm; (M,N) 50 μm; (O,P) 50 μm.

**Figure S6. Comparison of the distribution of Cx37 with integrin-α9 and Cx43 with ephrinB2.** (A-D) Whole-mount immunolabeling of mesentery from a P4 WT mouse. (A) Cx37 staining is concentrated at the valve (arrow) in the mesenteric collecting Ly. (B) Integrin-α9 staining of the same section as in (A) shows that integrin-α9 is also concentrated at the valve. (C) Merged image shows Cx37 (green) and integrin-α9 (red). (D) A higher magnification image of the valve in (C). Cx37 and integrin-α9 immunosignals overlapped in the valve, particularly towards the base of the valve. (E) Transverse section of a TD valve from a WT adult mouse immunostained for Cx37 (green) and integrin-α9 (red). Cx37 and integrin-α9 are both enriched in the valve and partially overlap there. (F) Section of a Ly vessel in the intestinal submucosa of a WT E18.5 embryo immunostained for Cx43. Cx43-containing punctae are marked by arrows. (G) The same section as in (F) immunostained for ephrinB2. EphrinB2 localizes to punctae (arrows) containing Cx43. Scale bars: (A-C) 50 μm; (D) 25 μm; (E) 10 μm; (F,G) 10 μm.

**Figure S7. Peripheral lymphatic patterning is normal in Cx37-/- and Cx37-/-Cx43+/- ear.** (A-F) Whole-mount immunolabeling of ear from adult mice with LYVE-1 antibody, which strongly labels the Ly capillaries. Patterning of Ly capillaries in Cx37-/- (B) and Cx37-/-Cx43+/- (C) ears looks similar to WT (A). (D-F) Higher magnification images of LYVE-1 stained Ly capillaries. (G-I) Whole-mount CD31 staining shows valves (arrows) in WT (G), Cx37-/- (H), and Cx37-/-Cx43+/- (I) collecting lymphatics of the ear. (J) The number of Ly capillary branch points/area (± s.e.m.) was determined for WT and Cx37-/-Cx43+/- whole-mount LYVE-1 stained ears. There was no significant difference between the means. The number of independent mouse samples analyzed is indicated (N). Scale bars: (A-C) 200 μm; (D-F) 200 μm; (G-I) 100 μm.

**Figure S8. Distribution of valves in the TD of a representative WT mouse.**

The schematic shows the location of four valves that were detected during serial sectioning of one representative WT adult mouse TD. Valves were found only in the rostral half of the TD and were more closely spaced toward the top.

**Figure S9. Smooth muscle actin expression is normal in Cx37-/-Cx43+/- TD.**

(A) Immunolabeling of transverse section of TD from an adult WT mouse for SMA (green) and Prox1 (red). Note that SMA staining is not continuous around the periphery of the TD. (B) SMA staining in the Cx37-/-Cx43+/- TD looks similar to WT. Scale bar: (A,B) 20 μm.

**Figure S10. Sporadic lymphatic phenotypes noted in Cx37-/-Cx43+/- or Cx37-/- adult mice.**

(A) Blood in the TD (td) of a Cx37-/-Cx43+/- mouse which was earlier injected with EBD for lymphangiography. The EBD is visible together with blood in the TD. The image is of the surface of a cryoblock during sectioning. (B) Dilated TD in a Cx37-/-Cx43+/- mouse. (C-D) Serial sections showing atresia of the TD in a Cx37-/-Cx43+/- mouse. The TD gets smaller and smaller and then abruptly ends (D). (E) Sharp right angle turn (arrow) in the TD of a Cx37-/-Cx43+/- mouse. In this case the TD does not continue upward along the midline; in other examples, the TD makes a sharp turn but also continues along the midline. (F) TD on the left side of the midline instead of the typical placement of the right side of the midline. (G) Grossly overgrown and convoluted TD valve (asterisk) in a Cx37-/- mouse. (H) Abnormal fat deposition around the heart (arrow) in a Cx37-/-Cx43+/- mouse. (I) Abnormal fat accumulation (arrows) around the aorta and TD in a Cx37-/-Cx43+/- mouse. (J) Abnormal cell aggregate in the lumen of the TD in a Cx37-/-Cx43+/- mouse. (K) An adjacent section to (J) immunostained for CD45 identifies the aggregated cells as leukocytes. (L) An adjacent section to (J,K) stained for F4/80 (red, macrophages) and CD3 (green, T cells). Scale bars: (B) 100 μm; (C,D) 100 μm; (G) 50 μm; (J) 20 μm; (K,L) 20 μm.

**Figure S1. Ultrastructure of a TD valve from an adult WT mouse shows that the valve is made up of two endothelial cell layers.**

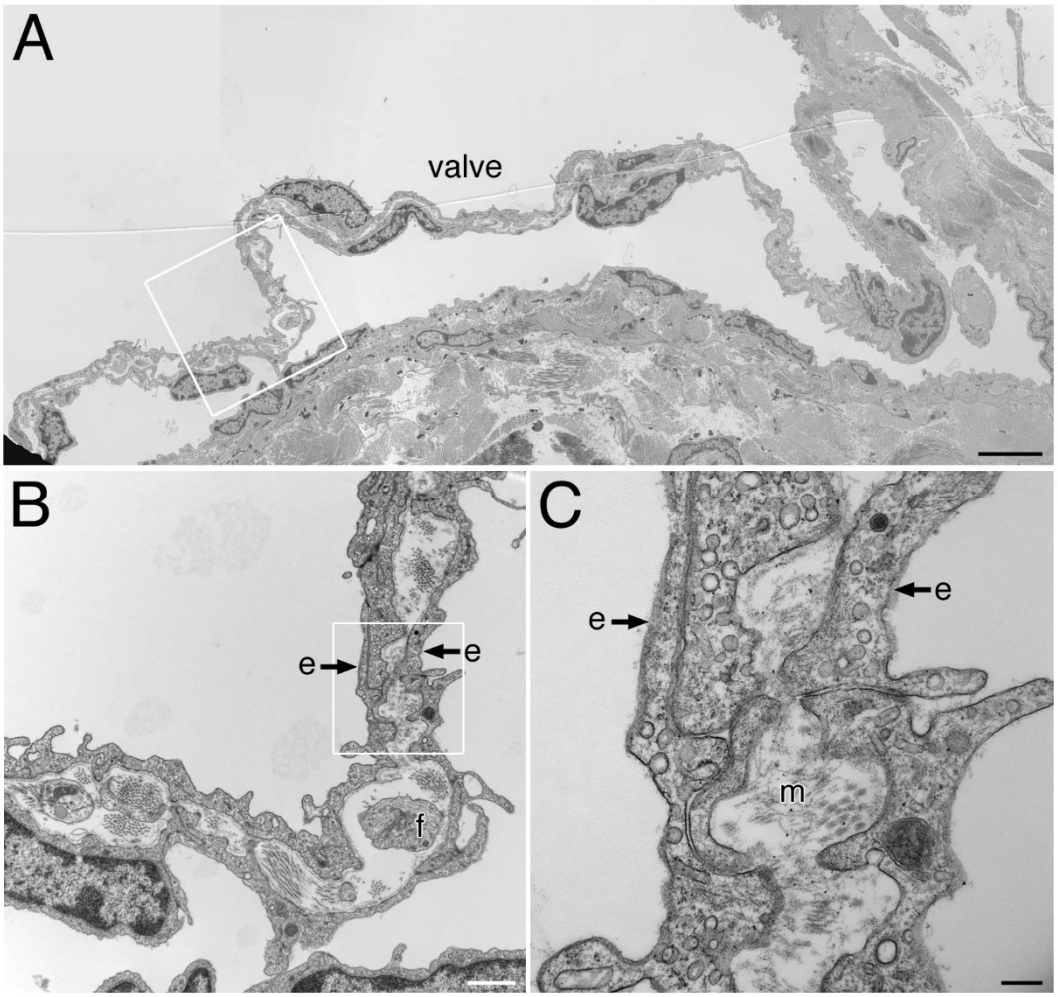


**Figure S2. Summary of Cx37, Cx43, and Cx47 expression in mature valved lymphatic vessels.**

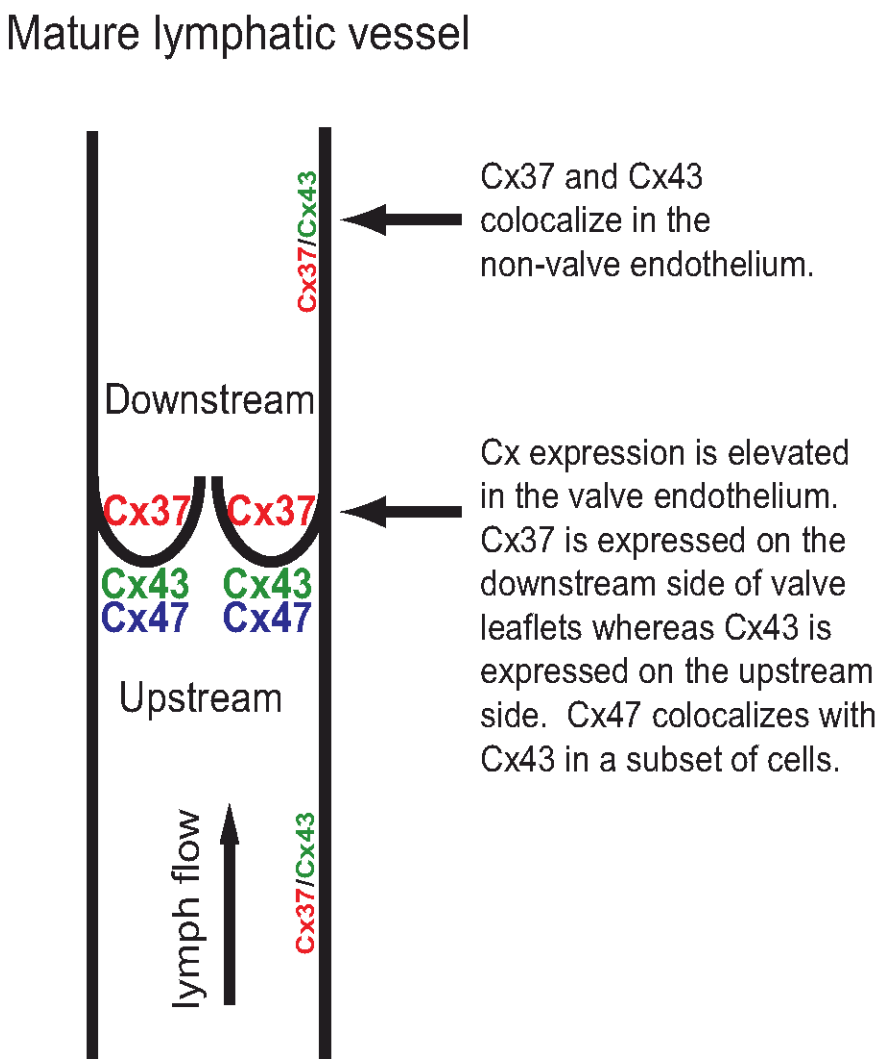


**Figure S3. Cx43 is required for valve development in intercostal lymphatic trunks.**

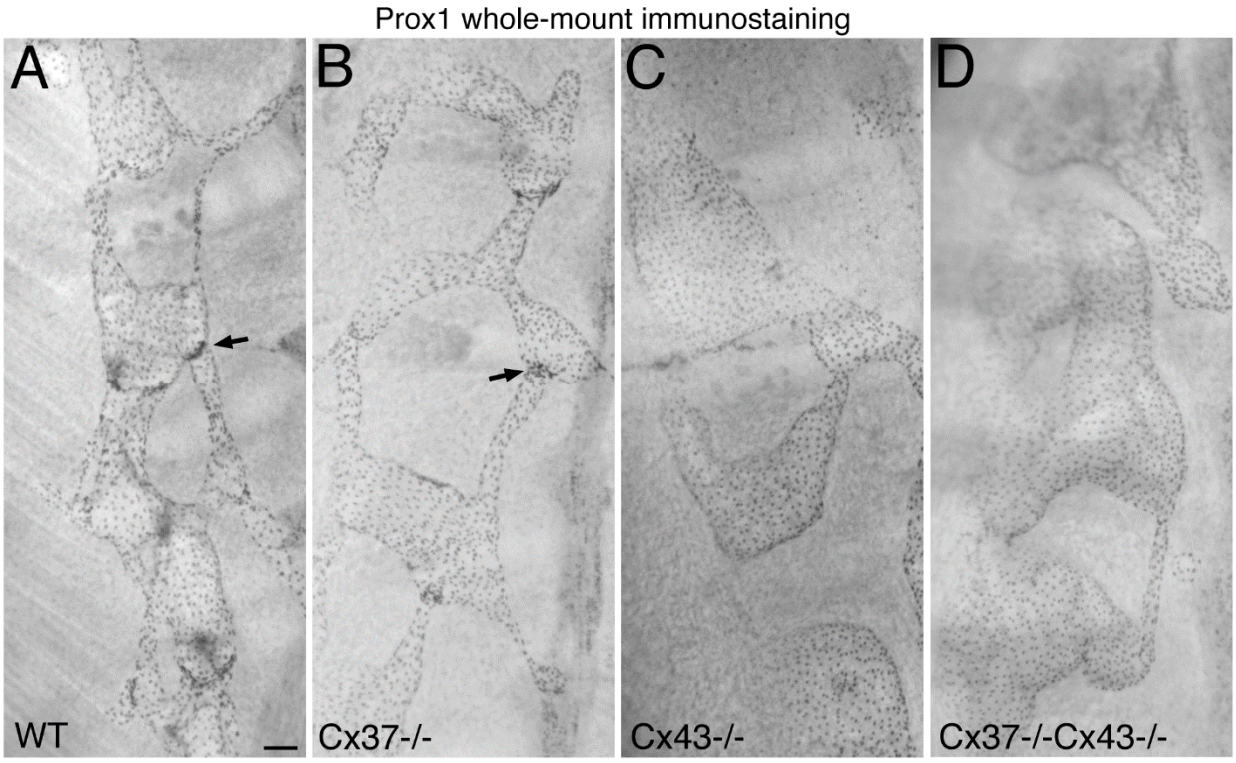


**Figure S4. Cx37-/-Cx43-/- E18.5 embryos lack valves in collecting lymphatics of the skin.**

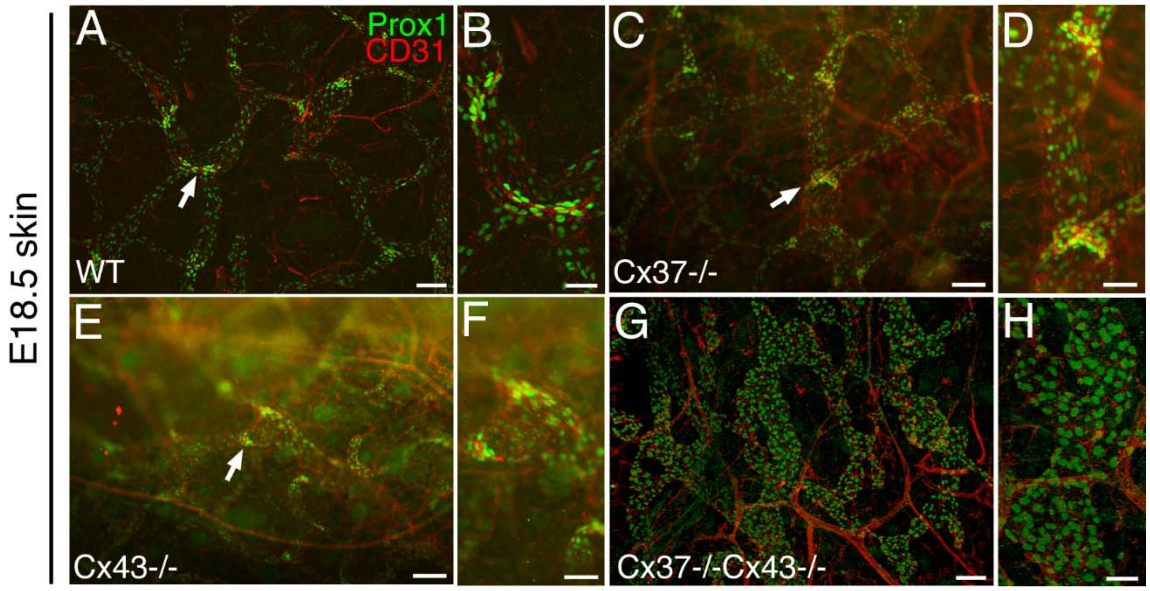


**Figure S5. Cx-deficient mesenteric lymphatics express appropriate markers of collecting vessel development.**

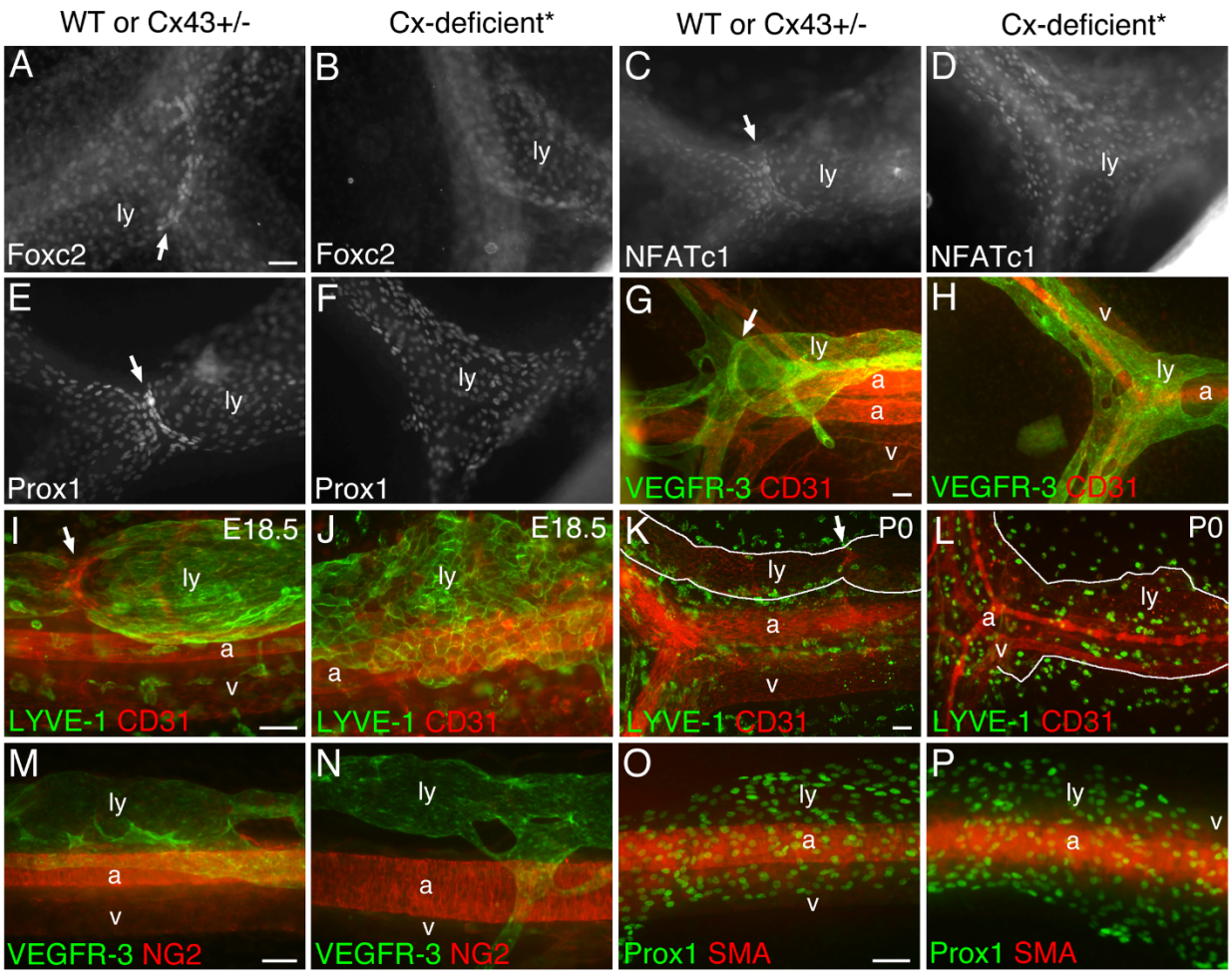


**Figure S6. Comparison of the distribution of Cx37 with integrin-α9 and Cx43 with ephrinB2.**

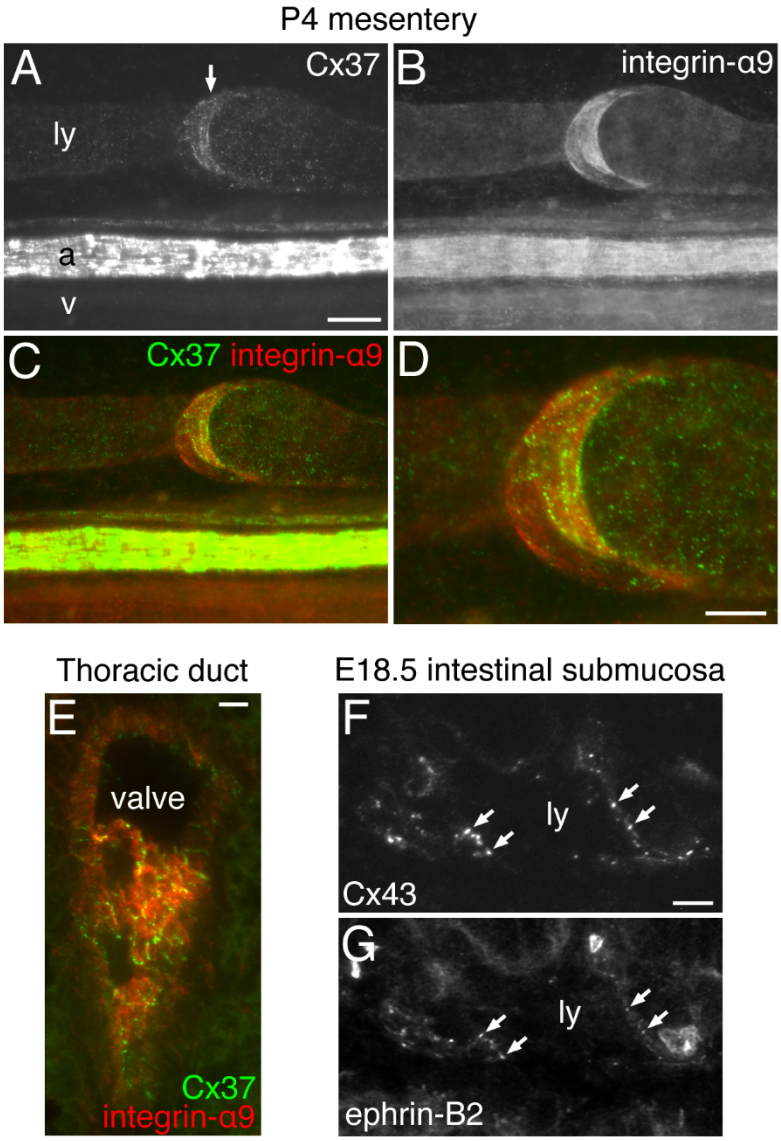
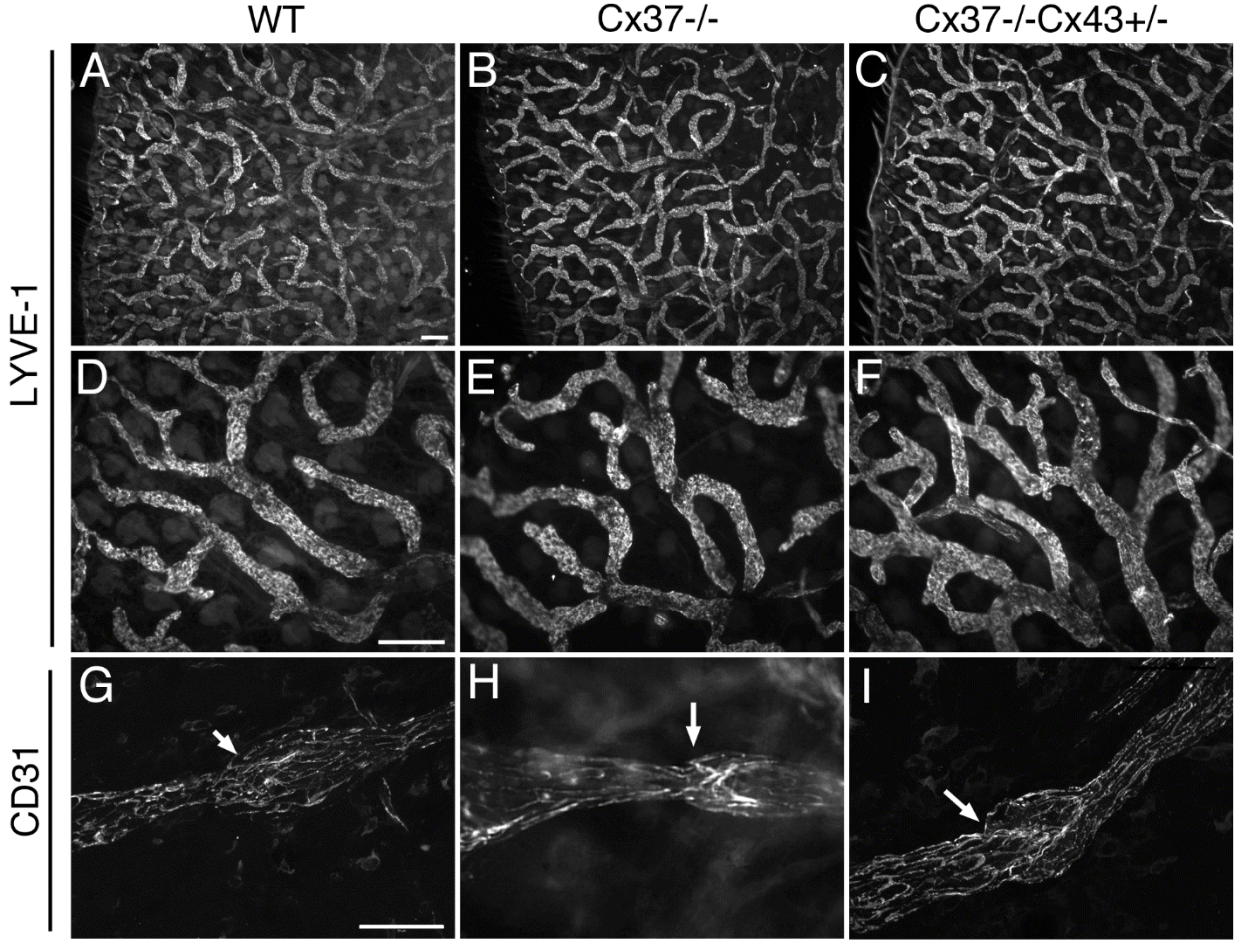


**Figure S7. Peripheral lymphatic patterning is normal in Cx37-/- and Cx37-/-Cx43+/- ear.**




J

| Genotype | N | Branch points/area |
|---|---|---|
| WT | 4 | 16.6 ± 1.3 |
| Cx37-/-Cx43+/- | 3 | 18.0 ± 1.7 |

**Figure S8. Distribution of valves in the TD of a representative WT mouse.**

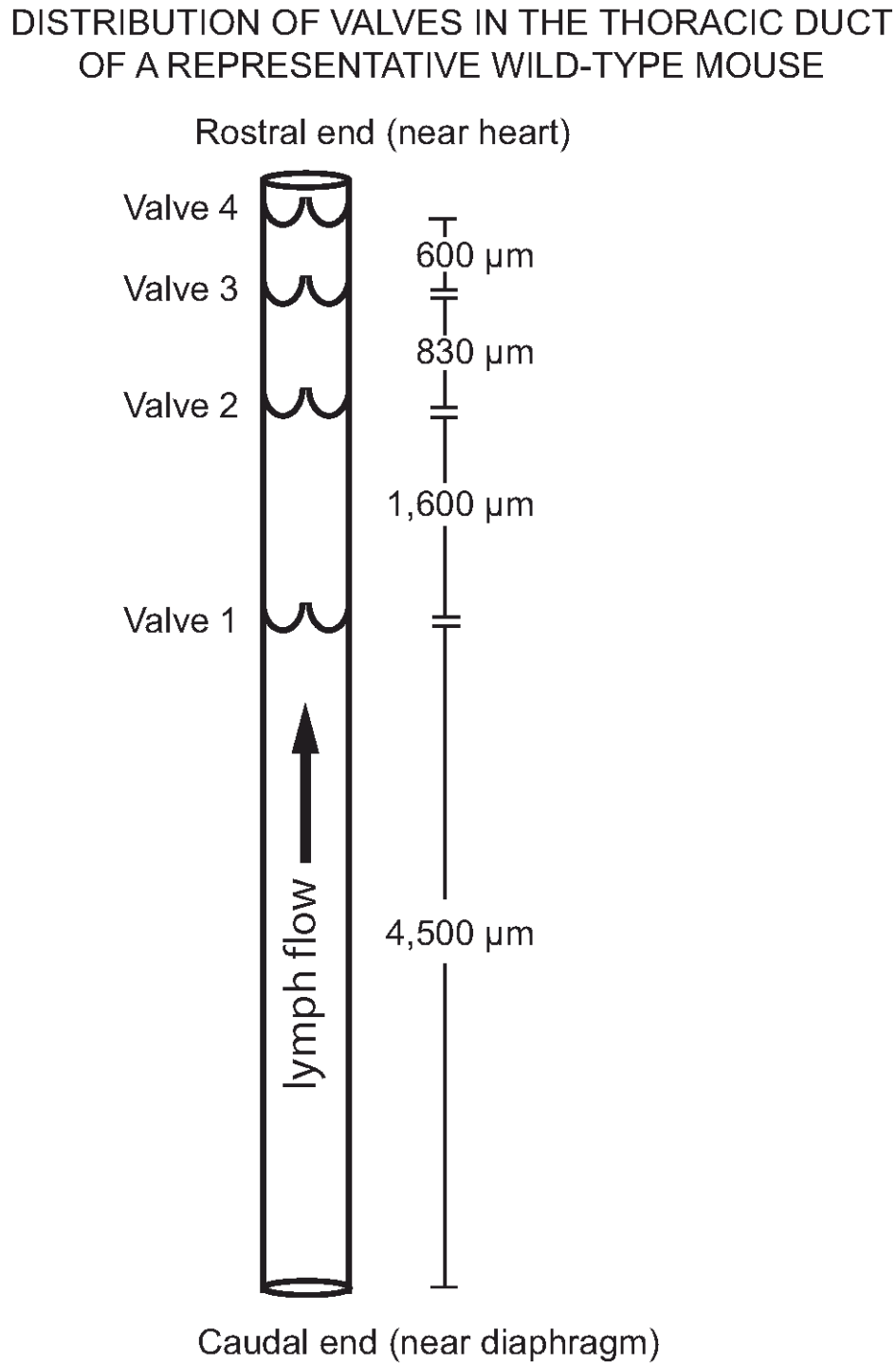


**Figure S9. Smooth muscle actin expression is normal in Cx37-/-Cx43+/- TD.**

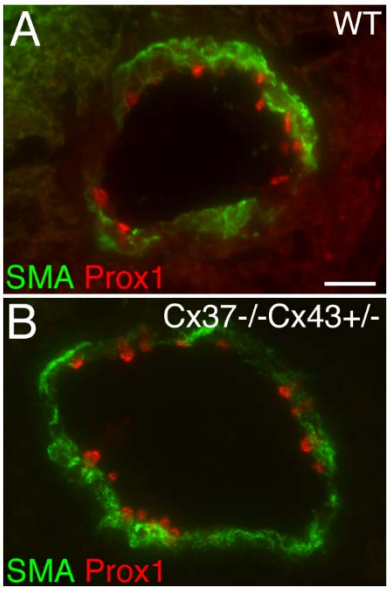


**Figure S10. Sporadic lymphatic phenotypes noted in Cx37-/-Cx43+/- or Cx37-/- adult mice.**

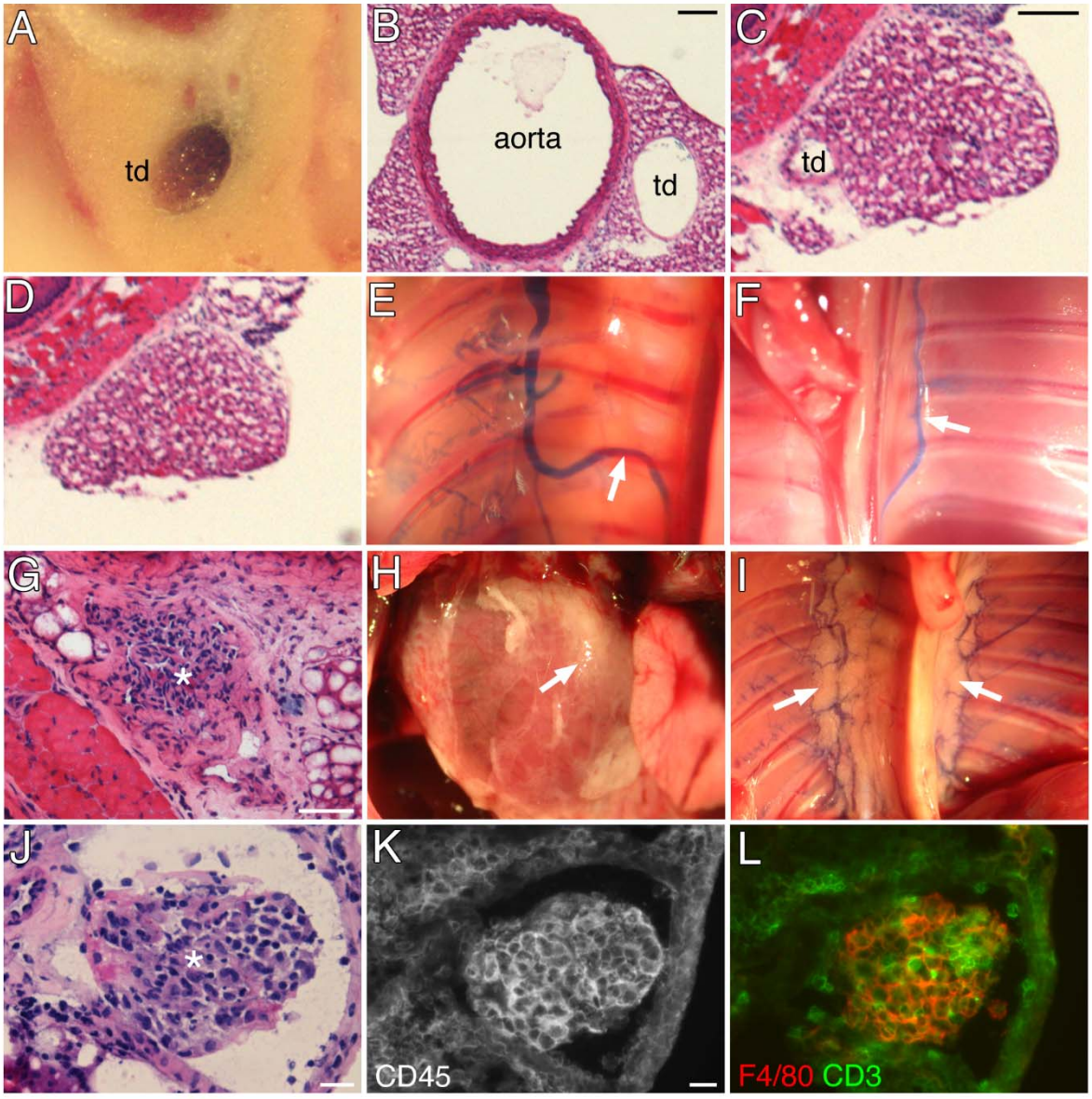


**Table S1. Frequency of lymphatic defects in E18.5-P0 Cx-deficient mice**

| Genotype (E18.5-P0) | Edema | | Blood in skin lymphatics | | Blood in mesenteric lymphatics | | Abnormal TD morphology | | Major Branches in TD | | Deficient diaphragm lymphatics | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **WT** | 0/157 | (0%) | 0/157 | (0%) | 0/157 | (0%) | 0/20 | (0%) | 3/20 | (13%) | 0/18 | (0%) |
| **Cx37+/-** | 2/51 | (3.9%) | 0/51 | (0%) | 2/51 | (3.9%) | 1/10 | (10%) | 1/10 | (10%) | 0/8 | (0%) |
| **Cx43+/-** | 0/64 | (0%) | 0/64 | (0%) | 1/64 | (1.6%) | 0/6 | (0%) | 0/6 | (0%) | 1/6 | (17%) |
| **Cx37+/-Cx43+/-** | 1/47 | (2.1%) | 1/47 | (2.1%) | 4/47 | (8.5%) | 0/6 | (0%) | 1/6 | (17%) | 1/8 | (13%) |
| **Cx37-/-** | 1/26 | (3.8%) | 0/26 | (0%) | 1/26 | (3.8%) | 1/10 | (10%) | 5/10 | (50%) | 0/9 | (0%) |
| **Cx43-/-** | 0/64 | (0%) | 0/64 | (0%) | 1/64 | (1.6%) | 6/6 | (100%) | 1/6 | (17%) | 4/6 | (67%) |
| **Cx37+/-Cx43-/-** | 14/44 | (32%) | 12/44 | (27%) | 13/44 | (30%) | 4/4 | (100%) | 2/4 | (50%) | 3/5 | (60%) |
| **Cx37-/-Cx43+/-** | 3/69 | (4.3%) | 2/69 | (2.9%) | 6/69 | (8.7%) | 7/13 | (54%) | 8/13 | (62%) | 2/12 | (17%) |
| **Cx37-/-Cx43-/-** | 28/42 | (67%) | 26/42 | (62%) | 25/42 | (60%) | 7/7 | (100%) | 3/7 | (43%) | 3/5 | (60%) |

The number of animals with the indicated phenotype per total number of animals analyzed as well as the percentage is presented in the table.

**Table S2. Genotypes of offspring at weaning age resulting from two types of crosses between mice deficient in Cx37 and Cx43.**

| | ♂ $Cx37^{+/-}Cx43^{+/-}$ ⊗ ♀ $Cx37^{+/-}Cx43^{+/-}$ crosses | | | ♂ $Cx37^{-/-}Cx43^{+/-}$ ⊗ ♀ $Cx37^{+/-}Cx43^{+/-}$ crosses | | |
|---|---|---|---|---|---|---|
| **Genotype** | **No. with genotype at weaning** | **Actual %** | **Expected* %** | **No. with genotype at weaning** | **Actual %** | **Expected* %** |
| **$Cx37^{+/+}Cx43^{+/+}$** | 79 | 8.9 | 8.3 | 0 | 0 | 0 |
| **$Cx37^{+/+}Cx43^{+/-}$** | 131 | 14.8 | 16.7 | 0 | 0 | 0 |
| **$Cx37^{+/-}Cx43^{+/+}$** | 185 | 20.9 | 16.7 | 123 | 20.7 | 16.7 |
| **$Cx37^{+/-}Cx43^{+/-}$** | 349 | 39.4 | 33.3 | 260 | 43.8 | 33.3 |
| **$Cx37^{-/-}Cx43^{+/+}$** | 74 | 8.4 | 8.3 | 99 | 16.7 | 16.7 |
| **$Cx37^{-/-}Cx43^{+/-}$** | 67 | 7.6 | 16.7 | 111 | 18.7 | 33.3 |
| **Total** | 885 | | | 593 | | |

* Expected % is calculated taking into account that mice with a $Cx43^{-/-}$ genotype are not viable to weaning age. With both types of crosses, $Cx37^{-/-}Cx43^{+/-}$ offspring at weaning are present at lower numbers than expected if there were no reduction in viability.

**Table S3. Genotypes of offspring at E18.5 resulting from crosses between mice deficient in Cx37 and Cx43.**

| | ♂ $Cx37^{-/-}Cx43^{+/-}$ ⊗ ♀ $Cx37^{+/-}Cx43^{+/-}$ | | |
|---|---|---|---|
| **Genotype** | **No. with genotype at E18.5** | **Actual %** | **Expected %** |
| **$Cx37^{+/-}Cx43^{+/+}$** | 34 | 12.1 | 12.5 |
| **$Cx37^{+/-}Cx43^{+/-}$** | 75 | 26.6 | 25 |
| **$Cx37^{+/-}Cx43^{-/-}$** | 43 | 15.2 | 12.5 |
| **$Cx37^{-/-}Cx43^{+/+}$** | 18 | 6.3 | 12.5 |
| **$Cx37^{-/-}Cx43^{+/-}$** | 75 | 26.6 | 25 |
| **$Cx37^{-/-}Cx43^{-/-}$** | 37 | 13.1 | 12.5 |
| **Total** | 282 | | |

**Table S4. Frequency of lymphatic defects in adult Cx-deficient mice and WT mice**

| Phenotype | WT | Cx43+/- | Cx37+/- Cx43+/- | Cx37-/- | Cx37-/- Cx43+/- | Cx40-/- |
|---|---|---|---|---|---|---|
| **Chylothorax** | 0/29 (0%) | 0/7 (0%) | 0/9 (0%) | 0/24 (0%) | 5/24 (21%) * | 0/9 (0%) |
| **Dye reflux into skin lymphatics of injected leg** | 0/21 (0%) | 0/7 (0%) | 1/9 (11%) | 12/15 (80%) | 24/24 (100%) | 0/9 (0%) |
| **Dye reflux into intercostal lymphatics** | 0/21 (0%) | 0/7 (0%) | 0/9 (0%) | 15/24 (63%) | 13/21 (62%) | 0/9 (0%) |
| **Dye reflux into mesenteric lymph nodes** | 1/22 (4.5%) | 0/7 (0%) | 1/9 (11%) | 0/12 (0%) | 7/12 (58%) | 0/9 (0%) |
| **Sharp turn or branch in the thoracic duct** | 0/29 (0%) | 0/7 (0%) | 1/9 (11%) | 5/24 (21%) | 3/20 (15%) | 0/9 (0%) |
| **Thoracic duct on the left side instead of right side** | 0/29 (0%) | 0/7 (0%) | 1/9 (11%) | 4/24 (17%) | 2/20 (10%) | 1/9 (11%) |
| **Dilated thoracic duct** | 0/5 (0%) | n.d. | n.d. | 1/5 (20%) | 4/10 (40%) | n.d. |
| **Thoracic duct atresia** | 0/5 (0%) | n.d. | n.d. | 0/5 (0%) | 2/10 (10%) | n.d. |
| **Blood in thoracic duct** | 0/5 (0%) | n.d. | n.d. | 0/5 (0%) | 3/10 (30%) | n.d. |
| **Cell aggregates in thoracic duct** | 0/5 (0%) | n.d. | n.d. | 1/5 (20%) | 3/10 (33%) | n.d. |
| **Deficiency in thoracic duct valves** | No, See Fig. 10 | n.d. | n.d. | Yes, See Fig. 10 | Yes, See Fig. 10 | n.d. |
| **Convoluted thoracic duct valve** | 0/5 (0%) | n.d. | n.d. | 1/5 (20%) | 0/5 (0%) | n.d. |
| **Abnormal fat deposition around heart or aorta** | 0/29 (0%) | 0/7 (0%) | 0/9 (0%) | 1/24 (4.2%) | 8/29 (28%) | 0/9 (0%) |

The number of animals with the indicated phenotype per total number of animals analyzed as well as the percentage is presented in the table. *The frequency of mice with chylothorax in the table represents only those mice that were analyzed by EBD lymphangiography. The actual frequency of chylothorax (with variable age of onset) in the Cx37-/-Cx43+/- population is much higher. n.d., not determined.

# APPENDIX B: COMBINING FOXC2 AND CONNEXIN37 DELETIONS IN MICE LEADS TO SEVERE DEFECTS IN LYMPHATIC VASCULAR GROWTH AND REMODELING

The following article is unpublished. It has been submitted to the journal, *Developmental Biology*, for review.

# Combining Foxc2 and Connexin37 deletions in mice leads to severe defects in lymphatic vascular growth and remodeling

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Short title: Combined loss of Foxc2 and Cx37 in lymphatic development

Keywords: Cx37, Foxc2, lymphatic valve, vascular development, lymphedema, lymphangiectasia

## Abstract:

Connexins (Cxs) play a critical role in lymphatic development. These proteins are vital for intercellular communication in vertebrates, but our knowledge is limited regarding their functional relationship with other proteins and signaling pathways. Recently, data from studies done in cell culture have shown that Cx37 is necessary for the coordinated activation of the transcription factor NFATc1, which cooperates with Foxc2 (another transcription factor) during lymphatic endothelial development. These data suggest that Cxs, Foxc2, and NFATc1 are part of a common developmental pathway. Here, we present a characterization (both macroscopic and microscopic) of Foxc2+/-Cx37-/- mice, which demonstrates that lymphatic network architecture and valve formation rely crucially on the concurrent embryonic expression and function of Foxc2 and Cx37. Foxc2+/-Cx37-/- mice have lymphedema *in utero*, exhibit craniofacial abnormalities, show severe dilation of intestinal lymphatics, display abnormal lacteal development, lack lymphatic valves, and typically die perinatally (outcomes not seen in Foxc2+/- or Cx37-/- mice separately). We provide a rigorous, quantitative documentation of lymphatic vascular network changes that highlight the specific structural alterations that occur in Foxc2+/-Cx37-/- mice. The lymphedema, intestinal lymphangiectasia, and facies observed in these mice are similar to the complications seen in patients with Hennekam syndrome, a rare congenital disorder. These data may thus provide fresh insight into the etiology of Hennekam syndrome and offer a new genetic target for screening and/or therapeutic exploration to treat this disease.

## Introduction:

Lymph vessels in humans form an extensive, hierarchical network that serves the body by executing a number of key functions: the trafficking of immune cells, absorption of dietary lipids, and maintenance of extracellular fluid balance. A spectrum of diseases is directly related to dysfunction of the lymphatic system. Complications with immune cell trafficking are associated with inflammatory and autoimmune disease, obstruction of the intestinal lymph vessels can cause protein-losing enteropathy, and the inability of the lymphatic system to accommodate protein/fluid loads leads to lymphedema. In addition, the lymphatic vasculature serves as a route of metastasis for many cancers. Greater emphasis on understanding lymphatic function has recently arisen due to the appreciation that a number of pathological states involve the lymphatic vasculature (Alexander et al., 2010; Wang and Oliver, 2010). Furthermore, various human congenital diseases involve defects in the architecture of the lymphatic system arising during embryologic development, with deleterious consequences projecting into early postnatal life and adulthood.

Lymphatic vascular development and function have become burgeoning fields of study, and experiments in recent years have shed light on numerous factors and events that govern the growth, remodeling, maturation, and function of the lymphatic vasculature (Koltowska et al., 2013). According to the prevailing model, early lymphatic development in the mouse begins with the onset of Prox1 expression in localized clusters of blood endothelial cells (BECs) within the cardinal vein at embryonic day (E) 9.5, an event associated with those cells adopting a lymphatic endothelial cell (LEC) identity. Those LECs then migrate away from the vein to form the lymph sacs and primary lymphatic plexus. The primary lymph plexus remodels through further sprouting, branching, and vessel fusion that establishes a hierarchy of lymph vessels throughout the tissues of the body. These vessels can be divided into two broad groups –

absorptive vessels (lymph capillaries) and conducting vessels (pre-collectors, collectors, and lymph trunks). Much attention has been focused on identifying the molecular players responsible for orchestrating this developmental course. Specific transcription factors (e.g. Sox18, COUP-TFII, Prox1, Foxc2, NFATc1), cell surface receptors (e.g. Tie2, VEGFR3, neuropilin, plexin A1, ephrin-B2, β1-integrin) as well as their ligands (e.g. Ang-2, VEGF-C, semaphorins, EphB4, fibronectin), and downstream effectors are part of the ensemble responsible for shaping the lymphatic vasculature (for a review, see Koltowska et al., 2013).

FOXC2, a **f**orkhead b**ox**, winged-helix transcription factor expressed by mesoderm and neural crest derived tissues, is essential for lymphatic development. Defective lymphatic remodeling, failure to form lymphatic valves, and abnormal mural cell coverage of lymphatic vessels have been found in *Foxc2-/-* mice (Petrova et al., 2004). *Foxc2-/-* mice also have severe problems with cardiovascular, ocular, and skeletal development and die embryonically/perinatally (Iida et al., 1997; Winnier et al., 1997). On the other hand, *Foxc2+/-* mice are viable, but have lymphatic valve insufficiency, distichiasis (double row of eyelashes), and dysmorphogenesis of lymph nodes (Kriederman et al., 2003; Shimoda et al., 2011). As such, these mice have been found to be a relevant model for lymphedema-distichiasis (LD), an autosomal dominant disorder in humans that typically presents as distichiasis and the pubertal onset of lymphedema in the lower limbs (Online Mendelian Inheritance in Man [OMIM] 153400). Mutations in the coding region for *FOXC2* have been linked to this disorder (Fang et al., 2000), but some cases of LD have been identified in non-coding regions (Sholto-Douglas-Vernon et al., 2005; Witte et al., 2009).

The current study focuses on the interplay during lymphatic development between Foxc2 and connexins, a family of proteins best known for their ability to mediate intercellular communication by forming channels (termed gap junction channels) that directly link the cytoplasm of neighboring cells. Connexins can also form hemichannels (“half” of a gap junction

channel) that allow the passage of substances between the cytoplasm and extracellular space. Three connexin isoforms (Cx37, Cx43, and Cx47) have been found in the murine lymphatic vasculature, and deletions or mutations in these isoforms cause lymphatic defects in mice and/or humans (Brice et al., 2013; Ferrell et al., 2010; Kanady et al., 2011). Lymphatic valve (Kanady et al., 2011; Sabine et al., 2012) and venous valve deficiencies (Munger et al., 2013) have been found in *Cx37-/-* mice. *Cx43-/-* mice are neonatal lethal and have cardiac malformations (Reaume et al., 1995), but they also lack lymphatic valves at E18.5 (Kanady et al., 2011). Combining genetic deficiencies in both *Cx37* and *Cx43* results in chylothorax as evidenced by the phenotype of *Cx37-/-Cx43+/-* mice, while *Cx37-/-Cx43-/-* embryos have lymph-blood mixing and develop lymphedema *in utero* (Kanady et al., 2011). In humans, late-onset lymphedema has been reported in a patient with oculodentodigital syndrome (ODD; OMIM 164200) – a disease affecting the face, eyes, teeth, and fingers – which is caused by mutations in *CX43* (Brice et al., 2013). *CX47* mutations in humans have also been linked to lymphedema (Ferrell et al., 2010).

From the studies above, disruption of lymphatic valve development/function was revealed as a commonality between mice lacking Foxc2, Cx37, or Cx43. Moreover, Foxc2 regulates (either directly or indirectly) Cx37 expression in LECs (Kanady et al., 2011; Sabine et al., 2012). ChIP-chip data has revealed a Foxc2 binding site in the vicinity of the *Cx43* gene (Norrmén et al., 2009), though previous data examining Foxc2-/- mice have shown no effect on Cx43 expression in LECs (Kanady et al., 2011). To further investigate the relationship between Foxc2 and connexins in lymphatic vascular development and function, we generated mice that have a *Foxc2* deficiency in conjunction with a deficiency of either *Cx37* or *Cx43*. In addition to Foxc2 being a regulator of Cx37 expression, our findings show that the function of Foxc2 during lymphatic vascular development also depends on the expression of Cx37. This is evidenced by

the phenotype of *Foxc2+/-Cx37-/-* mice, which bears similarity to the range of defects that occur in some lymphatic disorders, particularly Hennekam syndrome.

## Materials and Methods:

**Mice:**

*Cx37-/-* (*Gja4-/-*) (Simon et al., 1997), *Cx43-/-* (*Gja1-/-*) (Reaume et al., 1995), and *Foxc2-/-* (Iida et al., 1997b) lines were maintained on a C57BL/6 background and genotyped by PCR using previously published methods (Kanady et al., 2011). *Cx37+/-*, *Cx43+/-*, and *Foxc2+/-* mice were interbred to generate mice deficient in both Cx37 and Foxc2 or Cx43 and Foxc2. Animal protocols were approved by the IACUC Committee at the University of Arizona (Tucson, AZ).

**Antibodies:**

Primary antibodies used for immunostaining were as follows: rabbit antibodies to Cx37 (Simon et al., 2006), Cx43 (C6219, Sigma), Prox1 (11-002, AngioBio; ab11941, Abcam), pHH3 (06-570, Millipore); rat antibodies to CD31 (550274, BD Biosciences); goat antibodies to Foxc2 (ab5060, Abcam), Vefgr3 (AF743, R&D Systems). AffiniPure minimal cross reactivity secondary antibodies (conjugated to Alexa 488, Cy3, Cy5, or Dylight 649) were from Jackson Immunoresearch.

**Section immunostaining:**

Tissues were frozen unfixed in Tissue-tek O.C.T. and sectioned at 10 µm. Sections were fixed in acetone at -20°C for 10 minutes, blocked in a solution containing PBS, 4% fish skin gelatin, 1% donkey serum, 0.25% Triton X-100, and incubated with primary antibodies for 1.5 to 3 hours at room temperature or overnight at 4°C. Sections were washed with PBS containing 0.25% Triton X-100 and then incubated with secondary antibodies for 30-45 minutes. After washing, sections were mounted in Mowiol 40-88 (Aldrich) containing 1,4-diazobicyclo-(2,2,2)-

octane and viewed with an Olympus BX51 fluorescence microscope fitted with a Photometrics CoolSnap ES2 camera.

**Whole-mount immunostaining:**

Intestines (with mesentery attached) were fixed in 1% paraformaldehyde overnight at 4°C, washed in PBS, permeabilized with PBS containing 0.3% Triton X-100 overnight, and then blocked overnight in solution containing PBS, 4% fish skin gelatin, 1% donkey serum, 0.25% Triton X-100. Primary antibodies diluted in PBS containing 0.3% Triton X-100 were applied to the tissue overnight at 4°C. After washing, fluorescently labeled secondary antibodies were incubated overnight at 4°C. Following final washes, the mesenteries were mounted on slides in Citifluor mountant (Electron Microscopy Sciences). Skin tissue was treated similarly. Whole-mount samples were viewed with an Olympus BX51 fluorescence microscope fitted with a Photometrics CoolSnap ES2 camera or viewed with a Zeiss LSM 510 confocal microscope.

**Lymphangiography with Evans blue dye:**

Mice were anesthetized with an intraperitoneal injection of sodium pentobarbital or ketamine/xylazine mixture and kept warm. Evans blue dye (EBD) (1% w/v) was injected intradermally into the hindpaws and a dissecting microscope was used to examine EBD transport. Evidence of abnormal dye reflux into hindlimb skin or mesenteric lymph nodes was noted if present. The thoracic cavity was then opened and the presence of EBD in the TD was noted if present along with any abnormal dye reflux into intercostal Ly vessels. EBD was also injected into the ear to examine EBD transport in the dermal lymph network.

**Quantification of lymphatic vascular networks:**

Maximum intensity projections were generated from confocal z-stacks of whole-mount immunostained intestine and skin samples. Regions of intestine or skin that contained blood in the lymphatics were not included in the analysis as these could have been dilated due to luminal volume expansion. Manual image segmentation of the lymphatic vessels was then carried out based on Prox1 and CD31 or Vegfr3 immunolabeling using Adobe Photoshop CS4. The resulting segmentation was compared against the individual confocal slices used to create the maximum intensity projection to ensure accuracy. ImageJ was used to further process segmented images, and a skeletonization of the lymphatic network was created using the Skeletonize (2D/3D) plugin and measured using the AnalyzeSkeleton plugin (Arganda-Carreras et al., 2010). Segmented images were loaded into AngioTool (Zudaire et al., 2011) for quantitation of lacunarity (determined through box counting). Single factor ANOVA was performed for multi-wise comparisons, with post hoc Tukey-Kramer tests when ANOVA yielded a significant result ($p < 0.05$).

## Results:

### *Foxc2+/-Cx37-/-* mice exhibit generalized edema, craniofacial abnormalities, and die perinatally

In previous work using *Foxc2-/-* mice, we found that Cx37 expression was still present in the arterial vasculature, but was nearly absent in lymphatic endothelial cells (LECs) of the jugular lymph sac (a structure that forms during early lymphatic development) and greatly reduced in LECs of the mesentery. This finding suggested that *Cx37* gene expression was a target of regulation by Foxc2 in LECs (Kanady et al., 2011). Furthermore, recent experiments (Sabine et al., 2012) have provided mechanistic insight into LEC responses to fluid flow, and based on those data a model has been proposed whereby mechanotransduction, Prox1, and Foxc2 are involved in the control of Cx37 and calcineurin/NFAT (nuclear factor of activated T-cells) signaling in lymphatic valve development. We hypothesized that if Foxc2 controlled Cx37 expression, then combining deficiencies in these two proteins would recapitulate or exacerbate the lymphatic abnormalities seen in mice with deficits in either protein alone. To investigate this hypothesis, we surveyed mouse embryos at embryonic day (E) 18.5 for gross phenotypic differences among the following genotypes: *Cx37+/-*, *Cx37-/-*, *Foxc2+/-*, *Foxc2+/-Cx37+/-*, and *Foxc2+/-Cx37-/-*. *Foxc2+/-Cx37+/-* embryos were similar in appearance to wild type (WT), *Cx37+/-*, *Cx37-/-*, and *Foxc2+/-* embryos (Fig. 1A-D). Conversely, *Foxc2+/-Cx37-/-* embryos had an overall swollen appearance (indicative of generalized edema), with the extremities of these animals also affected (Fig. 1E,J). In addition, blunting of the snout was noted as a common feature in *Foxc2+/-Cx37-/-* mice. Thus, while *Foxc2+/-Cx37+/-* embryos were overtly normal, lymphedema only became apparent when there was *Foxc2* haploinsufficiency in conjunction with the complete loss of *Cx37*.

Dysmorphic facial features and defects in skeletogenesis have been reported in *Foxc2-/-* mice, but *Foxc2+/-* mice have been described as overtly normal in these respects (Iida et al., 1997; Winnier et al., 1997). Given our initial observations that *Foxc2+/-Cx37-/-* embryos had blunted snouts, we performed a detailed morphometric analysis of the head in order to further characterize any craniofacial differences. The length of the snout, snout width, and interpupillary distance were measured and expressed as a ratio of the distance between lambda and bregma (points at the posterior and anterior fontanelles, respectively) in E18.5 mice (Fig. 2E). The snout length of *Foxc2+/-Cx37-/-* (0.850 +/- 0.005) embryos was 7% shorter than WT (0.910 +/- 0.014), 8% shorter than *Cx37+/-* (0.921 +/- 0.006), and 11% shorter than *Foxc2+/-Cx37+/-* (0.950 +/- 0.016) embryos. The snout of *Foxc2+/-Cx37+/-* (1.034 +/- 0.018) mice was 7% wider than WT (0.964 +/- 0.014) and 7% wider than *Cx37+/-* (0.965 +/- 0.006) embryos. There were no statistically significant changes in interpupillary distance (Fig. 2F). While craniofacial abnormalities have been associated with human cases of lymphedema-distichiasis, we were unable to find differences in *Foxc2+/-* mice compared to other genotypes using the parameters measured. Interestingly, craniofacial differences only manifested when Foxc2 hemizygosity was combined with the loss of one or both alleles of *Cx37* in the mice we surveyed.

We noticed aggregations of blood faintly visible through the skin of *Foxc2+/-Cx37-/-* mice at E18.5. Dissections of the embryos revealed that the inguinal lymph node, as well as the dermal lymphatic vessels surrounding it, contained blood (Fig. 3C-E). Within the mesentery and intestinal wall of the proximal small intestine, there was a network of blood-containing, dilated vessels in some embryos (Fig. 3G,H). Immunohistochemistry confirmed the lymphatic identity of these vessels. The degree and extent to which blood was observed in the lymphatic vessels of the skin and intestine varied from mild to severe. Since *Foxc2-/-* mice have been reported to have defects in the formation and patterning of the aortic arch (Iida et al., 1997; Winnier et al.,

1997), we also inspected the heart, aorta, and its distributary blood vessels in *Foxc2+/-Cx37-/-* mice. There were no discernible abnormalities in the aorta or the principal arterial branches arising from it compared to other genotypes. However, wide, blind-ended, blood-filled vessels were present on the surface of the heart, and were likely dilated pericardial lymphatic vessels (Fig. 3J,K). Thus, in *Foxc2+/-Cx37-/-* mice, lymph-blood mixing occurred in the skin, intestine, and pericardial lymphatics, and these mice showed signs of intestinal and pericardial lymphangiectasia at E18.5.

*Foxc2+/-Cx37-/-* mice collected at E13.5, E18.5 and postnatal day (P) 0 showed similar developmental progression compared to littermates. Most embryos collected at E18.5 and P0 attempted to breathe and responded to physical stimuli. However, only three animals of this genotype out of 494 total animals collected survived postnatally – one died at P1, one at two months, and one at seven months of age. The expected genotypic frequency of generating *Foxc2+/-Cx37-/-* mice based on the crosses performed was 1/8, or approximately 62 animals in this population size. Since litters were routinely genotyped between P18 to P21, the observed genotypic frequency indicates that while *Foxc2+/-Cx37-/-* mice are fully developed in general by birth, most die perinatally or before weaning age.

### ***Foxc2+/-Cx37-/-*** **mice have dilated submucosal lymph vessels in the small intestine and defects in mesenteric lymph vessel remodeling and maturation**

We performed whole-mount immunofluorescence staining of E18.5 intestines using antibodies against vascular endothelial growth factor receptor 3 (Vegfr3) as a lymphatic marker to visualize the intact intestinal lymphatic vasculature (Fig. 4A-H). Vegfr3 is expressed by both blood and lymphatic endothelial cells during early embryogenesis, but becomes primarily restricted to LECs later in development, where its activation is required for lymphangiogenesis (Kaipainen et al., 1995; Karkkainen et al., 2004). Confocal laser scanning microscopy was used

to optically section through the intestinal wall and evaluate the submucosal lymphatic network as well as lacteals. By E18.5, the submucosal lymphatic network in WT mice has formed a highly branched system of vessels mostly homogenous in diameter (Fig. 4A). In comparison, *Foxc2+/-Cx37-/-* samples had a striking derangement of the submucosal lymphatic vasculature, which formed a disorganized network of dilated, cavernous lymph vessels (Fig. 4G). These vessels were erratic in caliber, ranging from approximately 20 to over 250 microns in diameter. This variation in vessel caliber gave the network a mesh-like appearance with holes that differed greatly in size. The mean submucosal lymph vessel diameter of *Foxc2+/-Cx37-/-* (45.0 +/- 6.63 $\mu$m) mice was 158% greater than WT (17.5 +/- 1.15 $\mu$m) mice, 145% greater than *Foxc2+/-* (18.36 +/- 0.65 $\mu$m), and 125% greater than *Cx37-/-* (19.96 +/- 1.33 $\mu$m) mice (Fig. 4I). The submucosal lymphatic vascular area density (lymph vessel area/tissue area) was 69% greater in *Foxc2+/-Cx37-/-* (0.840 +/- 0.022) mice compared to WT (0.496 +/- 0.013) mice, 65% greater compared to *Foxc2+/-* (0.509 +/- 0.012) mice, and 43% greater compared to *Cx37-/-* (0.593 +/- 0.024) mice (Fig. 4J). Conversely, lymphatic vascular length density (vessel length/tissue area) in *Foxc2+/-Cx37-/-* (0.019 +/- 0.0026) mice was 35% less than WT (0.029 +/- 0.0014) mice, 32% less than *Foxc2+/-* (0.028 +/- 0.0003) mice, and 37% less than *Cx37-/-* (0.030 +/- 0.0011) mice (Fig. 4K). Additionally, there was a 57% decrease in branch point density (branch points/tissue area) in the submucosal lymphatics of *Foxc2+/-Cx37-/-* (173 +/- 45 branch points/mm$^2$) mice compared to WT (402 +/- 38 branch points/mm$^2$) mice, a 55% decrease compared to *Foxc2+/-* (383 +/- 19 branch points/mm$^2$) mice, and a 58% decrease compared to Cx37-/- (413 +/- 31 branch points/mm$^2$) mice (Fig. 4L). Regions of the proximal small intestine were the most affected, with distal segments of the small intestine and large intestine showing less morphological irregularity. We calculated the percentage of tissue that was within a particular distance of a lymphatic vessel, and the majority of the parenchyma in the small intestine was closer to a given vessel in *Foxc2+/-Cx37-/-* mice compared to WT, *Foxc2+/-*, and

*Cx37-/-* mice (Fig. S2). Overall, the distribution and patterning of the submucosal lymphatic vasculature in *Foxc2+/-Cx37-/-* mice was significantly altered, which likely translated to postnatal lymphatic functional deficits in the intestine.

Lacteals are specialized lymphatic capillaries found in the villi of the small intestine that are responsible for a large portion of dietary fat and lipid-soluble vitamin absorption. Localized regions of intense Vegfr3 immunostaining readily identified the locations of developing lacteals in E18.5 whole-mount stained intestines (Fig. 5A-H), which allowed us to quantify the lacteal density (lacteals/tissue area) as well as measure their length. We saw lacteals associated with nearly all the villi we examined, and the lacteal density for WT (107 +/- 10 lacteals/mm$^2$), *Foxc2+/-* (101 +/- 13 lacteals/mm$^2$), *Cx37-/-* (108 +/- 7 lacteals/mm$^2$), and *Foxc2+/-Cx37-/-* (87 +/- 3 lacteals/mm$^2$) mice were similar (Fig. 5I). However, the lacteals of *Foxc2+/-Cx37-/-* samples were often wider at the base and 38% shorter (101.8 +/- 13.1 $\mu$m) compared to WT (165.1 +/- 12.0 $\mu$m), 40% shorter compared to *Foxc2+/-* (169.0 +/- 2.6 $\mu$m), and 38% shorter compared to *Cx37-/-* (163.3 +/- 16.8 $\mu$m) samples (Fig. 5J). While lacteal density in *Foxc2+/-Cx37-/-* mice was not affected, the shorter lacteals of these mice may contribute to decreased postnatal lipid-based nutrient absorption.

We next turned our attention to the lymphatic vessels of the mesentery. By E18.5 in WT mice, the mesenteric lymph vasculature has formed a fine, valved network of vessels with even caliber (Fig. 6A,E). In contrast to this, most of the mesenteric lymph vessels of *Foxc2+/-Cx37-/-* mice were hyperplastic, and exhibited larger than normal vessels (Fig. 6D,H). Some of the enlarged vessels were relatively even in caliber, while others were cavernous with highly irregular caliber. These cavernous segments had a “string of beads” appearance in some instances. Overall, the mesenteric lymphatic network in these animals had a mesh-like character, with holes that were highly variable in size. The mesenteric lymphatics also showed regional variability in the severity of enlargement, with vessels of relatively normal caliber in

some lymphatics primarily near the intestinal-mesenteric border. The lymphatic developmental anomalies of *Foxc2+/-Cx37-/-* mice within the mesenteric lymphatics are therefore similar to those seen in the submucosal lymphatics of the intestine, namely a gross enlargement of vessels and the formation of an erratic, plexiform vasculature.

Based on prospero homeobox protein 1 (Prox1) immunostaining, we found that mesenteric LECs in *Foxc2+/-Cx37-/-* mice displayed nuclear pleomorphism, with notable variance in both size and shape of nuclei (Fig. S3). Prox1 is a nuclear transcription factor that has been found to be expressed by the lymphatic endothelium and is required for lymphatic development (Wigle and Oliver, 1999). Prox1 positive nuclei were, in general, spherical or ellipsoid in shape. However, a proportion of them showed aberrations around the nuclear border that deviated from the mostly smooth contour typically seen in the nuclei of WT LECs. Some nuclei even displayed a kidney or horseshoe-shaped appearance. In WT mice, as the mesenteric lymph collectors become more developed, the LECs and their nuclei take on a more elongated shape. By P0, most of the nuclei of LECs in first order mesenteric lymph collectors take on an ovoid shape in normal mice. In contrast, the majority of the nuclei of LECs in *Foxc2+/-Cx37-/-* mice were spherical. The abnormalities in nucleus shape of *Foxc2+/-Cx37-/-* LECs indicate an overall gross disturbance in cell proliferation and LEC polarity.

Normally, intraluminal valves begin to form in the mesenteric lymphatic collecting vessels around E15.5 and by E18.5 they are abundant (Kim et al., 2007). The mesenteric lymph vessels in *Foxc2+/-Cx37-/-* mice at E18.5 (Fig. 6D,H) and P0 (Fig. 6L,P) completely lacked valves, and we did not see localized Prox1 upregulation that typically demarcates valve-forming areas. Unlike the regional variability in overall lymphatic vessel architecture, the absence of valves was uniformly observed in these animals. The absence of mesenteric lymphatic valves has been described for *Foxc2-/-* mice (Petrova et al., 2004), and a reduction in the number of mesenteric lymphatic valves at E18.5 has been reported for *Cx37-/-* mice (Kanady et al., 2011;

Sabine et al., 2012). While lymphatic valve functional insufficiency has been documented in *Foxc2+/-* mice (Kriederman et al., 2003a), mesenteric valves were not quantified in that study. Our results demonstrate that mesenteric lymphatic valve development fails to initiate or is arrested in the earliest stages when mice have deficiencies in both Foxc2 and Cx37. Moreover, these findings suggest that Foxc2 and Cx37 have synergistic roles in lymphatic valve development.

**Loss of Cx37 increases the mitotic activity of LECs in the mesentery of E18.5 mice**

We previously reported that *Cx37-/-* embryos develop enlarged jugular lymph sacs at E13.5 (Kanady et al., 2011). Given the severe enlargement of lymphatics in *Foxc2+/-Cx37-/-* mice, we investigated LEC proliferation in the mesenteric lymph vessels of *Cx37-/-* mice at E18.5. Histone H3 is a protein that is phosphorylated nearly exclusively during M-phase, and has thus been characterized as a "true" mitotic marker (Hendzel et al., 1997; Li et al., 2005; Tapia et al., 2006). Whole-mount immunostaining of E18.5 mesenteries with antibodies directed against phosphorylated Histone H3 (pHH3) revealed that the percentage of mitotically active LECs from *Cx37-/-* mice was 61% higher (20.00 +/- 0.53%) compared to WT mice (12.46 +/- 0.23%) (Fig. 7). Thus, the loss of Cx37 results in increased growth of lymphatic vessels by affecting mitotic activity.

**Cx37 is expressed in the lacteals and submucosal lymph vessels of E18.5 mice**

The severe defects in the formation of the submucosal lymphatic vasculature prompted us to check for Cx37 expression in the lymph vessels serving the parenchyma of the intestines. We probed for Cx37 in the lymphatic vessels serving the intestines of E18.5 WT mice. Cx37 was present in the lacteals and submucosal lymphatics of both the small (Fig. 8A-F) and large

intestine (Fig. 8G-L). Cx37 signal was punctuate and located at the membranes of Vegfr3 positive LECs.

**The dermal lymphatics of *Foxc2+/-Cx37-/-* mice have abnormal patterning, lack lymphatic valves, and form lymphangiomas**

Whole-mount preparations of skin from E18.5 WT, *Foxc2+/-*, *Cx37-/-*, and *Foxc2+/-Cx37-/-* mice were immunostained for Prox1 and platelet endothelial cell adhesion molecule 1 (PECAM-1) in order to assess lymphatic vessel and valve development (Fig. 9A-O). The lymphatic network of the skin in *Foxc2+/-Cx37-/-* embryos was composed of fine vessels, smaller in caliber than WT, *Foxc2+/-*, and *Cx37-/-* samples, which was a stark contrast to the severe enlargement of the lymphatic vessels of the intestinal and mesenteric lymph vessels. However, lymphatic vessels in some regions of the skin in *Foxc2+/-Cx37-/-* mice were hyperplastic. The mean diameter of dermal lymph vessels was 39% smaller in *Foxc2+/-Cx37-/-* mice (16.6 +/- 0.1 $\mu$m) compared to WT (27.2 +/- 2.0 $\mu$m) mice, and *Foxc2+/-* (18.2 +/- 1.3 $\mu$m) mice had a mean lymph vessel diameter that was 33% smaller compared to WT mice (Fig. 9P). The dermal lymphatic vascular fraction in *Foxc2+/-Cx37-/-* (0.119 +/- 0.000) mice was 35% less than WT (0.184 +/- 0.012) and 27% less than *Cx37-/-* (0.164 +/- 0.019) mice (Fig. 9Q). Lacunarity (which evaluates vascular structural nonuniformity), was significantly higher in *Foxc2+/-Cx37-/-* (0.60 +/- 0.03) samples compared to WT (0.41 +/- 0.04), indicating that dermal lymph vessels in *Foxc2+/-Cx37-/-* mice have less homogeneous lymphatic coverage in the skin. We also measured tortuosity of lymph vessel segments, branch point density, and lymph vessel length density, but found no differences in these measures between genotypes. Together, these data demonstrate that the dermal lymph vessels in *Foxc2+/-Cx37-/-* mice were thinner overall (contributing to a lower lymph vascular area density) and had an uneven distribution throughout the skin.

Lymphatic valves were completely absent from both the ventral and dorsal skin of *Foxc2+/-Cx37-/-* samples, and localized regions of Prox1 upregulation were not evident in these animals (Fig. 9J-L). There were also fewer lymphatic valves in the skin of *Foxc2+/-* (4.2 +/- 0.4 valves/mm$^2$) and *Cx37-/-* (3.5 +/- 0.2 valves/mm$^2$) mice compared to WT (7.5 +/- 0.6 valves/mm$^2$) mice (Fig. 9T). Interestingly, there was regionally skewed reduction in valves/valve-forming regions in *Cx37-/-* mice, with significantly decreased valve density in the dorsal skin (2.0 +/- 0.1 valves/mm$^2$) compared to the ventral skin (5.1 +/- 0.5 valves/mm$^2$). Thus, similar to mesenteric lymph vessels, there was valve agenesis in the dermal lymphatic network of *Foxc2+/-Cx37-/-* mice at E18.5.

Curiously, despite the reduction in overall dermal lymphatic vessel caliber and vascular fraction in *Foxc2+/-Cx37-/-* mice at E18.5, we found that the ends of some lymphatic capillaries had grown extremely large (to approximately 200 – 500 microns in diameter). The growths were Prox1 positive, variable in size, and highly cellular (Fig. 9M). We found them in various locations within the skin – both ventral and dorsal. PECAM-1 staining tended to be stronger in these structures compared to surrounding lymphatic vessels (Fig. 9N). These results show that hemizygous loss of Foxc2 combined with the loss of both alleles of *Cx37* in mice promotes the formation of structures that resemble cystic lymphangiomas.

### ***Foxc2+/-Cx37+/-*** **and** ***Foxc2+/-Cx43+/-*** **adult mice display similar lymphatic drainage patterns compared to** ***Foxc2+/-*** **mice**

Lymphatic drainage and function have been previously evaluated in *Foxc2+/-* adult mice by using Evans blue dye (EBD) lymphangiography, which revealed generalized lymphatic vessel and lymph node hyperplasia in those animals (Kriederman et al., 2003). In our past analysis of *Cx37-/-* adult mice, most had EBD reflux in the lymph vessels of the skin and the intercostal lymphatics, which suggested insufficient valve function and an impairment in maintaining

unidirectional lymph flow (Kanady et al., 2011). Because *Foxc2+/-Cx37-/-* mice die perinatally, we were unable to assess adults of this genotype. However, *Foxc2+/-Cx37+/-* mice are viable and EBD lymphangiography was performed on adult mice of this genotype. Cx43 is necessary for normal lymphatic vascular development (Kanady et al., 2011) and results from ChIP-chip studies identified a Foxc2-NFATc1 binding site pair within 230 kilobases of the Cx43 gene (Norrmen et al., 2009). Therefore, we also assessed *Foxc2+/-Cx43+/-* adult mice. *Foxc2+/-Cx37+/-* and *Foxc2+/-Cx43+/-* adult mice (3 to 6 months of age) exhibited similar EBD drainage patterns in the ear compared to *Foxc2+/-* mice (Fig 10B-D). Hindpaw EBD injections in these mice also showed comparable drainage patterns in the skin to *Foxc2+/-* mice, and EBD was readily observed in the lymphatics serving the inguinal lymph node, lumbar lymph nodes, as well as the thoracic duct of both *Foxc2+/-Cx37+/-* and *Foxc2+/-Cx43+/-* mice (Fig. 10E-L). Reflux of EBD into the intercostals lymphatic network was not found, suggesting that the valves in *Foxc2+/-Cx37+/-* and *Foxc2+/-Cx43+/-* were competent under the fluid load of the EBD injections. In stark contrast to the severe developmental defects associated with the complete loss of Cx37 on a *Foxc2+/-* background, these functional assessments of lymphatic drainage demonstrate that the presence of a single copy of Cx37 is sufficient to largely preserve valve function in *Foxc2+/-Cx37+/-* mice.

## Discussion:

In this study, we extend the concept of a functional link between Foxc2 and Cx37 by demonstrating the significance of their combined loss, not only in terms of lymphatic valve development but also more broadly in the remodeling of both conductive and absorptive lymphatic vessels. We found that *Foxc2+/-Cx37-/-* mice have lymphedema *in utero*, develop craniofacial abnormalities, show drastic dilation of intestinal lymphatics, display abnormal lacteal development, fail to develop valves in the collecting vessels of the mesentery and skin, develop lymphangiomas, and typically die perinatally. In contrast to this, the lymphatic phenotype of *Foxc2+/-Cx37+/-* and *Foxc2+/-Cx43+/-* mice is similar to *Foxc2+/-* mice, albeit with the addition of subtle craniofacial differences in *Foxc2+/-Cx37+/-* mice compared to other genotypes. Our phenotypic characterization of *Foxc2+/-Cx37-/-* mice provides *in vivo* evidence that lends support to a model by which these two proteins jointly regulate lymphatic vascular development through a common molecular pathway.

### Foxc2 and Cx37 control of bone formation and function

The craniofacial differences that manifested only in *Foxc2+/-Cx37+/-* and *Foxc2+/-Cx37-/-* mice suggested that Foxc2 and Cx37 were mutual regulators of bone morphogenesis. Many lymphedema-associated disorders are accompanied by craniofacial and ocular defects (for a review, see Northup et al., 2003). It has been proposed that these defects are caused by the impaired migration of tissues during embryonic development due to fluid pressures exerted by lymphedema that arises *in utero* (Witt et al., 1987). However, craniofacial abnormalities can manifest from birth in humans with hereditary lymphedema, even when lymphedema is of late-childhood or adolescent onset (Fang et al., 2000). Thus, an alternative mechanism exists outside, or perhaps in conjunction with, the external influence of hydrostatic

forces to affect craniofacial development in inheritable lymphatic disorders. Indeed, while *Foxc2+/-Cx37-/-* embryos collected at E18.5 showed evidence of generalized lymphedema, intrauterine lymphedema was not observed in *Foxc2+/-Cx37+/-* mice. Nevertheless, both these genotypes displayed dysmorphic facial features, indicating that both lymphedema and non-lymphedema-dependent mechanisms affecting bone development may have been at play.

There are a number of similarities in the expression and function of Foxc2 and Cx37 in bone cells that point to a possible non-lymphedema-dependent mechanism that would explain the craniofacial differences in *Foxc2+/-Cx37+/-* and *Foxc2+/-Cx37-/-* mice. Foxc2 is expressed in bone marrow mesenchymal stem cells and preosteoblasts, where it is critical for osteoblast proliferation, differentiation, and survival (Park et al., 2011). Evidence for the importance of Foxc2 in bone development can be seen in the phenotype of *Foxc2-/-* mice, where in addition to problems with cardiovascular and lymphatic development, defects in skeletogenesis have been reported (Iida et al., 1997a). Cx37 is also expressed in bone cells (osteoblasts, osteocytes, and osteoclasts), and has an important influence on structural bone characteristics. Mice with a global deletion of *Cx37* have higher bone mineral density, trabecular bone volume, and mechanical strength. These changes were attributed to defective osteoclast differentiation associated with elevations in Notch signaling, a pathway that controls cell fate determination (Pacheco-Costa et al., 2014). Interestingly, single nucleotide polymorphisms (SNPs) in both *FOXC2* and *CX37* are associated with changes in bone mineral density (BMD) in Japanese populations. The C-512T *FOXC2* SNP is associated with reductions in BMD in Japanese men and women (Yamada et al., 2006), whereas the C-1019T *CX37* SNP is associated with reduced BMD in Japanese men (Yamada et al., 2007). These findings, together with our data regarding the development of craniofacial abnormalities in mice only when deficiencies in Foxc2 and Cx37 are combined, support the idea of complementary functional roles for these two proteins in bone formation. To further substantiate this idea, it would be interesting to screen the individuals in

the aforementioned studies for SNPs in both *FOXC2* and *CX37* (if the combination of these SNPs is compatible with survival) to determine if decreases in their BMD are exacerbated.

**Foxc2 and Cx37 in intestinal lymphangiogenesis**

Intestinal lymphangiectasia (pathological dilation of lymph vessels) in *Foxc2+/-Cx37-/-* mice signified a severe disturbance in lymphangiogenesis. At the turn of the 20$^{th}$ century, Heuer described the basic sequence of events associated with lymphangiogenesis in the intestinal lymphatic network. Intestinal lymphatic vessels emerge from the retroperitoneal lymph sac, following the mesenteric arteries as they lead into the intestinal wall. The vessels spread through an active branching process, merging together in the submucosa as they envelop the intestine (Heuer, 1909). The lymph vessels in *Foxc2+/-Cx37-/-* mice followed mesenteric blood vessels to establish the foundation of the lymphatic vascular network, indicating that basic guidance cues (and the ability to respond to them) during lymphangiogenesis were maintained. However, the profound dilation and decreased branch point density of intestinal lymphatics revealed a break down in the processes governing vessel sprouting, branching and/or anastomosis.

The regulation of the above processes by Notch signaling has been the center of interest in many efforts to elucidate the molecular pathways controlling angiogenesis. Interestingly, Dll4 (Delta-like ligand 4, a Notch receptor ligand) haploinsufficient mice have branching and vessel fusion defects in the retinal blood vasculature, resulting in the formation of a vascular plexus that looks similar to the submucosal lymphatic plexus of *Foxc2+/-Cx37-/-* mice. Recent studies have linked Notch signaling to the control of Cx37 expression. In cultured human dermal LECs, activation of Notch1 and Notch4 signaling dramatically increases *Cx37* mRNA transcripts and protein (Murtomaki et al., 2014). In LEC-specific *Notch1-/-* mice, downregulation of *Cx37* and enlarged lymph vessels have been reported. The enlargement of lymph vessels in *Notch1-/-*

mice was attributed to increased LEC proliferation and decreased LEC death (Fatima et al., 2014). In accordance, we also found increased proliferation of mesenteric LECs in *Cx37-/-* mice, which is consistent with studies reporting that Cx37 regulates cellular growth (Burt et al., 2008; Fang et al., 2011; Good et al., 2014). Additionally, Foxc2 can influence Notch signaling by directly activating the transcription Dll4 and therefore acts upstream of the Notch pathway (Hayashi and Kume, 2008; Seo et al., 2006). Together, these data suggest that the architectural changes to the submucosal lymphatics in *Foxc2+/-Cx37-/-* mice may be related to a role for Foxc2 and Cx37 in Notch signaling and in the control of cellular proliferation, branching, and vessel fusion.

Remarkably, lymphangiectasia in the intestine *Foxc2+/-Cx37-/-* mice was mainly localized to the proximal small intestine (near the pyloroduodenal junction), evidence of a region-specific impairment in lymph-vascular development. In contrast, lymphangiectasia was not present in the stomach or large intestine. During early embryonic development, three broad domains are established in the gut along the rostrocaudal axis that give rise to specific sections of the alimentary canal – the foregut (esophagus and stomach), midgut (small intestine), and hindgut (large intestine and rectum). The restriction of lymphangiectasia within the gastrointestinal tract to the small intestine suggests that Foxc2 and Cx37 have a greater influence on mid-gut derived lymphatic tissues compared to other areas. Developmental defects principally in the small intestine are also seen in *Foxl1* (also known as Fkh-6, another member of the forkhead-box family of transcription factors) knockout mice. In addition to intestinal epithelial defects, *Foxl1-/-* mice have problems with the formation of gut-associated lymphoid organs (GALT) – particularly in the development of Peyer's patches and colonic patches, which are specialized lymphoid follicles in the small and large intestine respectively (Fukuda et al., 2003). Foxl1 has localized expression in the mesoderm of the mid- and hindgut (Kaestner et al., 1997). Based on their tandem, clustered arrangement within a 10 kb region on

the same chromosome, it has been suggested that Foxl1 and Foxc2 share regulatory mechanisms (Kaestner et al., 1996).

The lacteals of *Foxc2+/-Cx37-/-* mice were considerably shorter, only spanning approximately half the length of the villus in many cases. Lacteals form part of the mucosal lymphatic network, which develops secondarily to the submucosal lymphatic network (Heuer, 1909). The incomplete development of lacteals in *Foxc2+/-Cx37-/-* mice reflects a shortcoming of the system to properly remodel the submucosal lymphatic network. Disruption of lacteal development was also demonstrated in mice lacking Angiopoietin-2 (Ang-2, a vascular growth factor), resulting in abnormally short lacteals or often their absence (Gale et al., 2002). Ang-2 has been shown to be under the control of Prox1 and Foxc2 in mouse embryonic-stem-cell-derived endothelial cells (Harada et al., 2009) as well as under the control of Foxc2 in adipocytes (Xue et al., 2008). These data, together with our findings, argue that Cx37 may be linked (potentially through Foxc2) to Ang-2 dependent pathways in the development of lacteals. Characterization of Cx37 expression and function in Ang-2 deficient mice will help determine the spatiotemporal relationships of these proteins.

Clinically, intestinal lymphangiectasia can give rise to chronic diarrhea, loss of serum proteins into the intestinal lumen (protein-losing enteropathy), and impaired absorption of fats and fat-soluble vitamins. Coupled with defective lacteal development, problems related to intestinal loss of serum proteins and nutrient malabsorption during critical postnatal periods may have been major contributing factors to death in *Foxc2+/-Cx37-/- mice*.

**Foxc2, Cx37, and Cx43 in lymphatic valve development**

Based on data showing that Cx45 in the cardiac endothelium was necessary for the activation of NFATc1 with epithelial-mesenchymal transformation during cardiac valve morphogenesis, it was suggested that connexins were involved in calcineurin/NFATc1 signaling

(Kumai et al., 2000). Supporting this suggestion, oscillatory shear stress experiments on LECs in culture have also shown that Cx37 is required for the coordinated activation of NFATc1 (Sabine et al., 2012). A link between NFATc1 and Foxc2 has been shown via chromatin immunoprecipitation data combined with tiling microarrays, which have identified Foxc2 binding sites within the genome that are in close proximity to predicted NFATc1 sites. Furthermore, luciferase reporter assays using fragments from some of those identified regions showed synergistic activation in the presence of both Foxc2 and NFATc1 (Norrmen et al., 2009). Additionally, a partial genetic interaction between Foxc2 and Cx37 was evinced by differences in valve maturation in *Foxc2+/-Cx37+/-* mice compared to *Foxc2+/-* and *Cx37+/-* mice. These findings have coalesced into a proposed model whereby mechanotransduction, Prox1, and Foxc2 are involved in the control of Cx37 and calcineurin/NFAT signaling in lymphatic valve development (Sabine et al., 2012). Our data concerning the absence of intraluminal valves in the lymphatic collectors of the skin and mesentery of *Foxc2+/-Cx37-/-* mice at E18.5 are not incompatible with this model. Connexin-based calcium signaling has been demonstrated to occur via intercellular diffusion of $Ca^{2+}$ or inositol trisphosphate through gap junctions (Boitano et al., 1992; Giaume and Venance, 1998) or by purinergic nucleotide-based paracrine mechanisms via hemichannels (Ponsaerts et al., 2010). Thus, one possible scenario in *Foxc2+/-Cx37-/-* mice is that the loss of Cx37 represents a reduced capacity for connexin-mediated calcium signaling in response to mechanical events, leading to decreased activation of calcineurin and lower levels of activated NFATc1. Coupled with a *prima facie* reduction in the levels of Foxc2 in Foxc2+/- mice, a subsequent decrease in Foxc2-NFATc1 cooperative regulation of gene expression might be expected in *Foxc2+/-Cx37-/-* mice.

Importantly, the phenotype of *Foxc2+/-Cx37-/-* mice reveals a number of new insights into the relation of these two proteins to lymphatic valve development. Since *Foxc2+/-* and *Cx37-/-* mice each have approximately a 50% reduction in the number of developing valves in

the collecting lymphatic vessels of the skin at E18.5, the complete loss of valves in *Foxc2+/-Cx37-/-* embryos points to an additive effect of Foxc2 and Cx37 on valve formation. Moreover, there was no evidence of localized upregulation of Prox1 (demarcating valve-forming areas) during lymphatic vessel remodeling in *Foxc2+/-Cx37-/-* mice, indicating that valve formation failed to initiate or was arrested at very early stages. On the other hand, *Cx37-/-* mice do show localized, circumferential upregulation of Prox1 during early lymphatic valve genesis, but often have defective valve maturation (Kanady et al., 2011; Sabine et al., 2012). These points are particularly interesting, because they reveal that in the absence of Cx37, a full complement of Foxc2 is needed to start the valve formation program. Thus, the function of Foxc2 (or that of the proteins that it transcriptionally regulates) depends on Cx37 expression.

In discussing mechanisms that lead to the initiation of valve formation, it is also important to consider that Cx43 is coexpressed with Cx37 in LECs prior to valve formation. Also, like *Foxc2+/-Cx37-/-* mice, there is a failure to locally upregulate Prox1 expression in LECs and to subsequently initiate valve development in *Cx43-/-* mice. Thus, the initiation of lymphatic valve formation depends on more than one connexin. One possibility is that the initiation of valve formation is a function of the overall level of intercellular coupling and the expression level of Foxc2. In this scenario, *Cx37-/-* mice would have decreased coupling, but due to the presence of Cx43 channels and a full complement of Foxc2, the threshold to initiate valve formation is achieved. However, in the case of *Foxc2+/-Cx37-/-* mice, the combined reduction in coupling and Foxc2 results in the inability to achieve the threshold for valve initiation. An additional nuance is that Cx37 and Cx43 are capable of forming heteromeric channels (composed of a mixture of Cx37 and Cx43 in a single channel), and heteromeric channels (in general) have distinct functional characteristics compared to their corresponding homomeric channels (those composed of either Cx37 or Cx43 alone) (Brink et al., 1997; Burt and Steele, 2003; Cottrell and Burt, 2001; Cottrell et al., 2002; Elenes et al., 2001; Heyman et al., 2009;

Martinez et al., 2002). Therefore, the connexin composition of channels in LECs should be taken into account when considering how these proteins influence the different stages of lymphatic valve development.

**Foxc2 and Cx37 in dermal lymphangiogenesis.**

The morphological changes (overall reduction in vessel caliber and lower density of coverage) in the peripheral lymphatic network of *Foxc2+/-Cx37-/-* mice in the skin run counter to that of the collecting vessels of the mesentery and intestine (increased vessel caliber and higher area density). Cx37 is expressed to a lesser extent in dermal lymphatic capillaries (Kanady et al., 2011) compared to lymphatic collecting vessels. This difference in expression level may reflect a functionally distinct role of Cx37 in the peripheral lymphatic vessels, explaining in part the developmental differences in peripheral versus central lymphatic development in *Foxc2+/-Cx37-/-* mice. However, consistent with vessel enlargement seen in the mesentery and intestine, portions of the dermal lymphatic network were also enlarged and structures resembling lymphangiomas developed in some of these mice.

The differing growth/remodeling effects of Foxc2 and Cx37 on specific levels of the lymphatic vascular hierarchy are unclear, but may be related to LEC versus non-LEC connexin expression and function. Cx37 is expressed by macrophages where it is involved in modulating ATP-dependent adhesion (Wong et al., 2006). Intriguingly, it has been shown that macrophages play a role in defining dermal lymphatic vascular caliber by limiting LEC proliferation (Gordon et al., 2010). Therefore, the loss of Cx37 in macrophages may be partly responsible for the changes in lymphatic vascular caliber seen in *Foxc2+/-Cx37-/-* mice. Cx37 is also expressed in the ectoderm of rats during embryonic development (Goliger and Paul, 1994). Interestingly, mice with ectodermal-specific ablation of Cx26 have defects in development of the dermal lymphatic vasculature (Dicke et al., 2011). Thus, cross-talk between

germ layers (ecto/meso/endoderm) mediated by connexins could represent an important mode by which peripheral lymphatic development is governed. Evaluating mice with tissue-specific deletion of *Foxc2* and/or *Cx37* may reveal the precise cellular domains in which these proteins function to control lymphatic growth and remodeling.

**Similarity of congenital lymphatic disorders in humans to that of *Foxc2+/-Cx37-/-* mice**

There are a number of human congenital diseases associated with both lymphedema and craniofacial abnormalities, such as lymphedema-distichiasis (LD), Noonan syndrome (OMIM 163950), and Hennekam syndrome (OMIM 235510). While the etiology of these diseases has been traced to particular genes in many instances, there is genetic heterogeneity in their manifestation. Noonan syndrome has eight sub-classifications - 1 through 8 – caused by mutations in different genes on different chromosomes (most frequently in *PTPN11*, *SOS1*, and *RAF1*). Patients afflicted with these subtypes have commonalities in their disease presentation, although with distinct variations in their phenotype. Mutations in *FOXC2* have been found to cause LD (Fang et al., 2000), but in genetic screenings of some families with LD, no mutations in *FOXC2* were found (Finegold, 2001). *CCBE1* (collagen and calcium-binding EGF domains 1) is a gene that encodes for an extracellular matrix protein essential for lymphangiogenesis (Bos et al., 2011) and mutations in *CCBE1* have been discovered in individuals with Hennekam syndrome (Alders et al., 2009; Connell et al., 2010). However, in an analysis of 22 families with Hennekam syndrome, only five families were found to carry mutations in *CCBE1* (Alders et al., 2009). Therefore, the frequency of Hennekam syndrome cases not associated with *CCBE1* mutations is demonstrative of genetic heterogeneity in the occurrence of this disorder. As our results demonstrate a genetic interaction between *Foxc2* and *Cx37* in both lymphatic vascular development and bone formation, it will be useful to

consider the involvement of these genes in cases of the above diseases where no mutation has been identified.

Hennekam syndrome is an autosomal recessive disease, which in addition to lymphedema and craniofacial abnormalities, is also characterized by lymphangiectasias and mental retardation (Hennekam et al., 1989). Interestingly, three of these defining symptoms were observed in Foxc2+/-Cx37-/- embryos - lymphedema, facies, and lymphangiectasia. As these mice almost always died perinatally, assessment of neurologic development was not possible. The similarities in the phenotype of *Foxc2+/-Cx37-/-* mice to patients with Hennekam syndrome are striking, though. Thus, genetic screenings for concurrent mutations in these two genes may reveal a multigenic etiology to Hennekam syndrome in some patients lacking *CCBE1* mutations. Alternatively, the similarity in phenotype may be due to an as yet unknown role for Ccbe1 in molecular pathways involving Foxc2 and/or Cx37.

**Summary**

This study shows that both Foxc2 and Cx37 are necessary for lymphangiogenesis to proceed correctly. A myriad of new lymphatic defects manifests when hemizygous deletion of Foxc2 is combined with complete ablation of Cx37 (compared to those resulting from their individual loss), suggesting that Foxc2 function depends on the presence of Cx37. Establishment of the primary lymphatic plexus occurs in Foxc2+/-Cx37-/- mice, but its remodeling into a functional vascular network replete with lymphatic valves is severely impaired. Foxc2+/-Cx37-/- mice also have dysmorphic cranial development, indicating that Foxc2 and Cx37 may have shared domains of control in bone development as well. The abnormalities that occur in Foxc2+/-Cx37-/- mice mirror the spectrum of symptoms seen in some lymphedema-associated disorders. Thus, considering a multigenic etiology for patients with lymphatic disorders may benefit future studies attempting to identify the genetic basis for these diseases.

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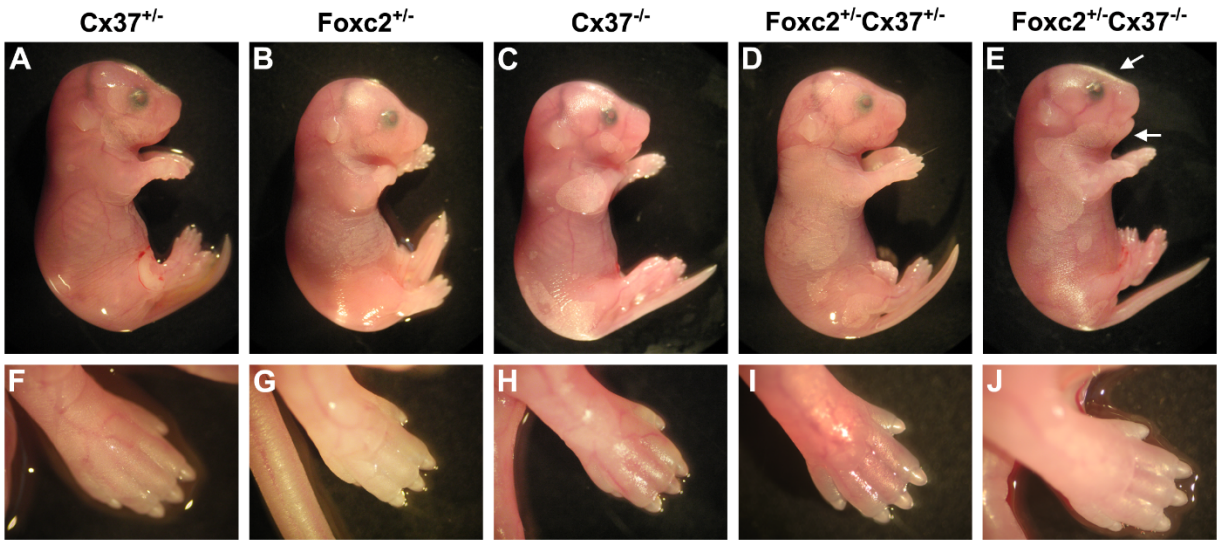


**Figure 1.** Gross images of E18.5 mice. Littermates with the following genotypes are shown: Cx37+/- (A,F), Foxc2+/- (B,G), Cx37-/- (C,H), Foxc2+/-Cx37+/- (D,I), and Foxc2+/-Cx37-/- (E,J). Lateral views of the whole embryo (A-E), with corresponding images of the hindlimb (F-J). Foxc2+/-Cx37-/- embryos presented with mild generalized edema (E) that also affected the hindlimbs (J). Flattening of the nasal bridge (E, upper arrow) and micrognathism (E, lower arrow) also occurred. No overt differences in gross embryonic morphology were notable among the other genotypes (A-D, F-I).

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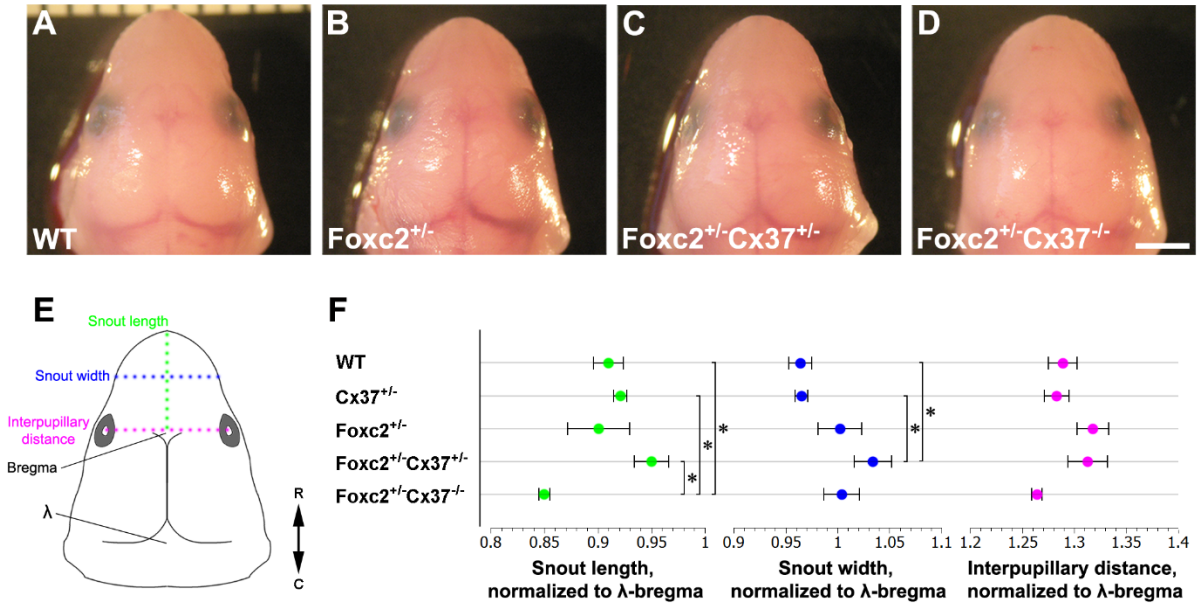


**Figure 2.** Craniofacial morphometrics of E18.5 embryos. (A-D) Superior view of the head of littermates with the following genotypes: WT (A), Foxc2+/- (B), Foxc2+/-Cx37+/- (C), and Foxc2+/-Cx37-/- (D). (E) Schematic diagram of the embryonic head indicating where snout length (green), snout width (blue), and interpupillary distance (magenta) were measured. Bregma and lambda (λ) are shown on the schematic at the anterior and posterior fontanelles, respectively. Double-headed arrow denotes R (rostal) and C (caudal) directions. (F) Numeric measures of snout length (green points), snout width (blue points), and interpupillary distance (magenta points) normalized to λ –bregma distance are graphed for WT, Cx37+/-, Foxc2+/-, Foxc2+/-Cx37+/-, and Foxc2+/-Cx37-/- mice. The length of the snouts of Foxc2+/-Cx37-/- mice were significantly shorter than WT, Cx37+/-, and Foxc2+/-Cx37+/- mice. The width of the snouts of Foxc2+/-Cx37+/- mice were significantly greater than WT and Cx37+/- mice. No statistically significant differences were found for interpupillary distance among genotypes analyzed. Scale bar: (A-D) 2 mm. Values are presented as means, with error bars indicating standard error of the mean. Asterisks, $p < 0.05$. Single factor ANOVA followed by post hoc Tukey-Kramer tests were performed for statistics.

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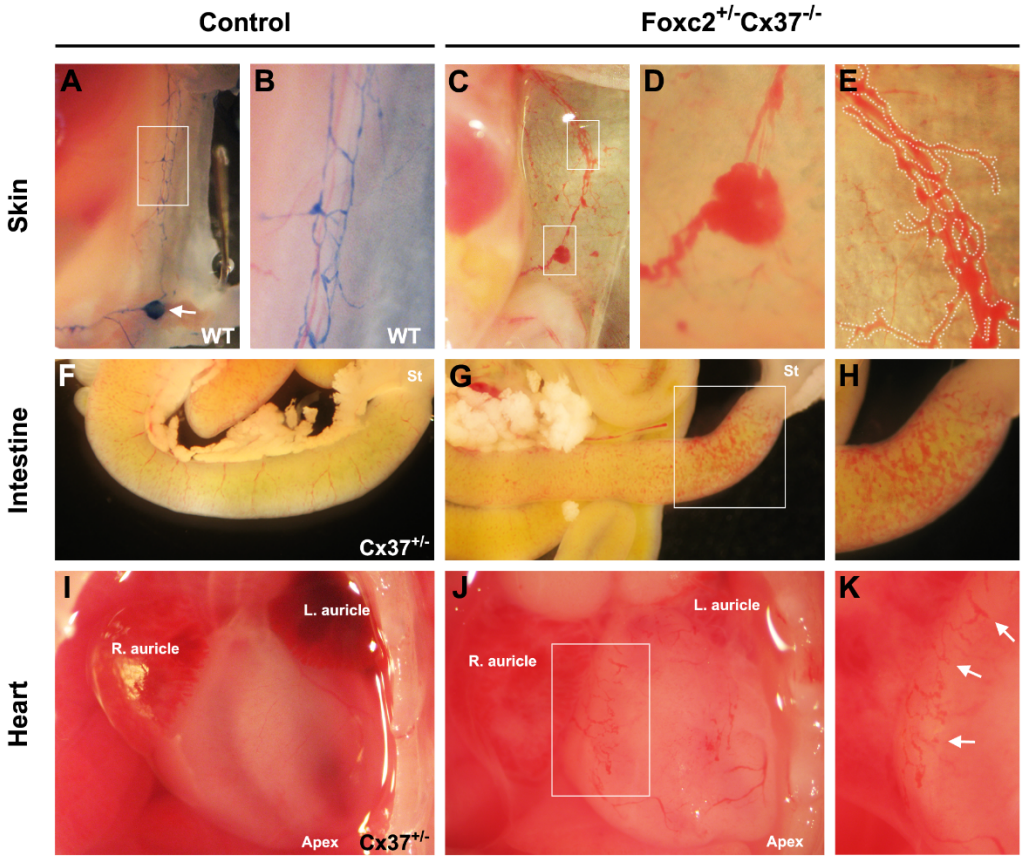


**Figure 3.** Lymph-blood mixing in the lymphatics of the skin (C-E), intestines (G,H), and heart (J,K) of Foxc2+/-Cx37-/- mice at E18.5. (A) Evans blue dye (EBD) injection of E18.5 WT embryo showing normal drainage pattern from the inguinal lymph node (arrow) and thoracoepigastric lymphatic vessels. (B) Thoracoepigastric lymph vessels, higher magnification view of the white box from (A); note the fine caliber of WT lymph vessels in this region. (C) Blood within the inguinal lymph node (lower box) and thoracoepigastric lymph vessels (upper box) of a Foxc2+/-Cx37-/- embryo. (D) Higher magnification view of lymph node from (C). (E) Higher magnification view of thoracoepigastric lymph vessels from (C); blood-filled lymph vessels are digitally outlined in white for better contrast. (F) Gross image of the small intestine from an E18.5 Cx37+/- control embryo, compared to a Foxc2+/-Cx37-/- littermate (G). (H) Higher magnification view of white box from (G); blood is present within the serosal lymphatics in the proximal small intestine. (I) Gross image of the heart from E18.5 Cx37+/- control embryo; right/left auricles and apex are labeled. (J) Blood accumulation in blind-ended vessels of the pericardium in an E18.5 Foxc2+/-Cx37-/- embryo. (K) Higher magnification view of white box from (J), arrows highlight blood-filled lymph vessels.

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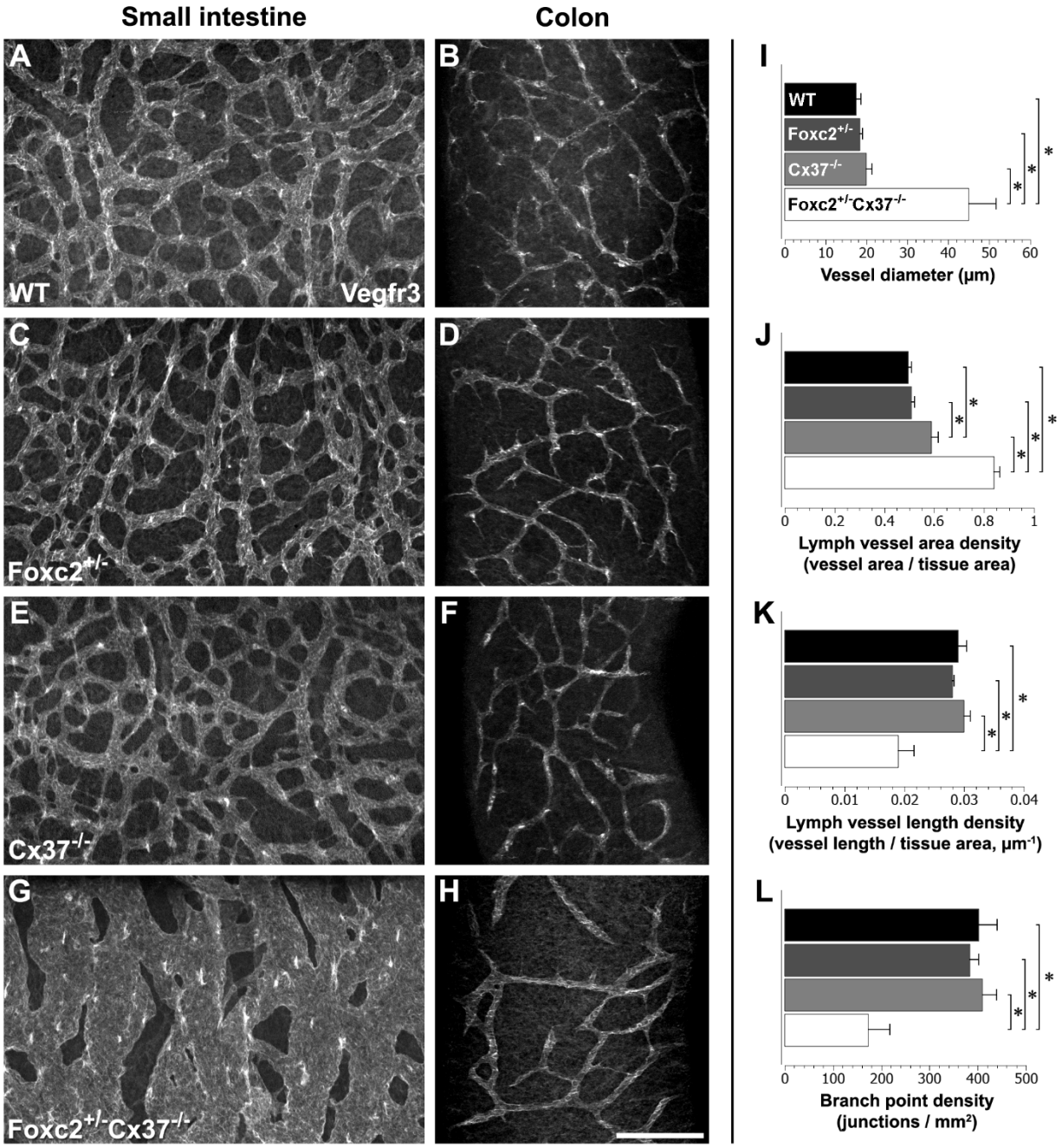


**Figure 4.** Characterization of intestinal lymphatics in WT, Foxc2+/-, Cx37-/-, and Foxc2+/-Cx37-/- mice at E18.5. Confocal immunomicrographs of the small intestine and colon from E18.5 WT (A,B), Foxc2+/- (C,D), Cx37-/- (E,F), and Foxc2+/-Cx37-/- mice are shown; samples were probed for Vegfr3. The lymphatic vessels were segmented based on Vegfr3 signal and skeletonized. (I-L) Quantitation of the submucosal lymphatic network of the proximal small intestine. Submucosal lymph vessel diameter (I)

and submucosal lymph vessel area density (I) of Foxc2+/-Cx37-/- mice were significantly greater than WT, Foxc2+/-, and Cx37-/- mice. Submucosal lymph vessel length density (K) and submucosal lymphatic branch point density (L) of Foxc2+/-Cx37-/- mice were significantly lower than WT, Foxc2+/-, and Cx37-/- mice. Additionally, submucosal lymph vessel area density of Cx37-/- mice was significantly greater than WT and Foxc2+/- mice. Scale bar: (A-H) 200 μm. Values are presented as means, with error bars indicating standard error of the mean. Asterisks, $p < 0.05$. Single factor ANOVA followed by post hoc Tukey-Kramer tests were performed for statistics.

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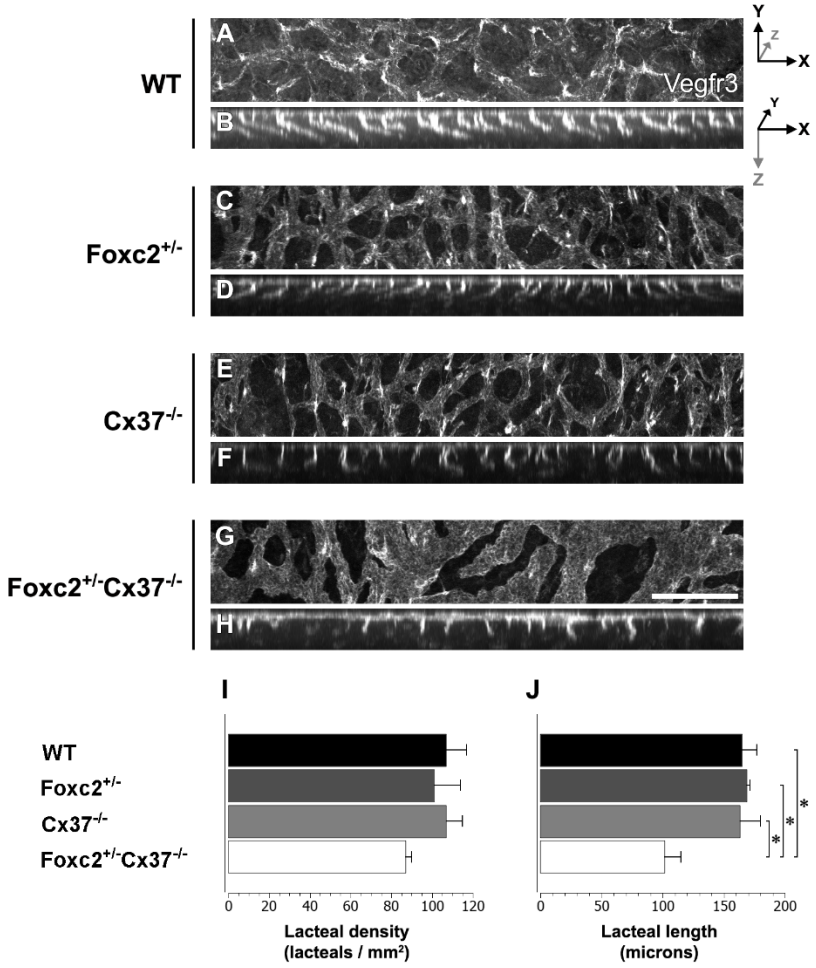


**Figure 5.** Characterization of lacteals in the proximal small intestine of E18.5 WT, Foxc2+/-, Cx37-/-, and Foxc2+/-Cx37-/- mice. Maximum intensity projections of confocal z-stacks in the X-Y and X-Z planes for WT (A,B), Foxc2+/- (C,D), Cx37-/- (E,F), and Foxc2+/-Cx37-/- mice; samples probed for Vegfr3. Measurements of lacteal density (I) and lacteal length (J) for WT (black bars), Foxc2+/- (dark grey bars), Cx37-/- (light grey bars), and Foxc2+/-Cx37-/- (white bars) mice are shown. Lacteals from Foxc2+/-Cx37-/- mice were significantly shorter than WT, Foxc2+/-, and Cx37-/- mice. Scale bar: (A-H) 200 µm. Values are presented as means, with error bars indicating standard error of the mean. Asterisks, $p < 0.05$. Single factor ANOVA followed by post hoc Tukey-Kramer tests were performed for statistics.

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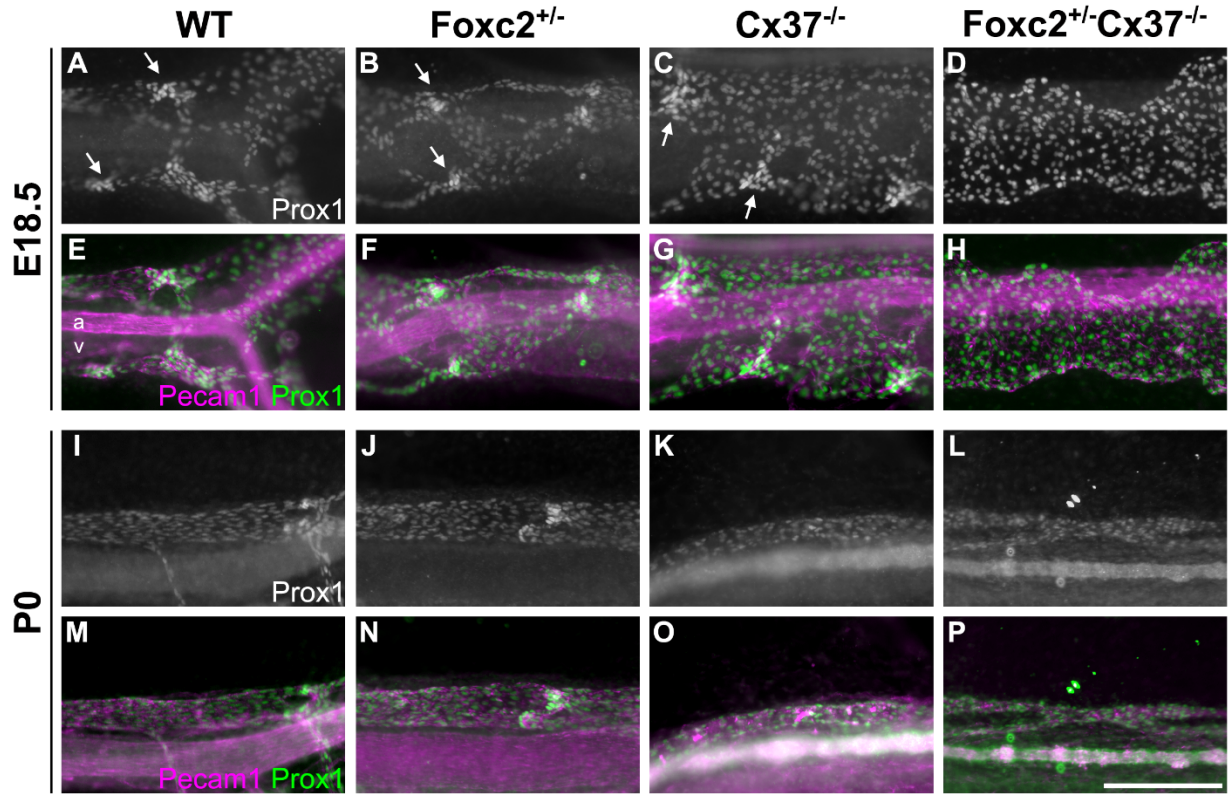


**Figure 6.** Mesenteric lymph vessels of Foxc2+/-Cx37-/- mice lack valves and are dilated at E18.5. Prox1 whole-mount immunostaining highlights lymphatic vessels of the mesentery in WT (A), Foxc2+/- (B), Cx37-/- (C), and Foxc2+/-Cx37-/- (D) mice at E18.5. Arrows in (A-C) denote lymphatic valve forming areas; note that none are present in Foxc2+/-Cx37-/- mice (D). Corresponding Prox1 (green) and Pecam1 (magenta) color composites are shown for WT (E), Foxc2+/- (F), Cx37-/- (G), and Foxc2+/-Cx37-/- (H) mesenteries at E18.5. (I-L) Prox1 whole-mount immunostaining at P0 for WT (I), Foxc2+/- (J), Cx37-/- (K), and Foxc2+/-Cx37-/- (L) mesenteries. (M-P) Prox1 (green) and Pecam1 (magenta) color composites are shown for WT (M), Foxc2+/- (N), Cx37-/- (O), and Foxc2+/-Cx37-/- (P) mesenteries at P0. Scale bar: (A-P) 200 µm.

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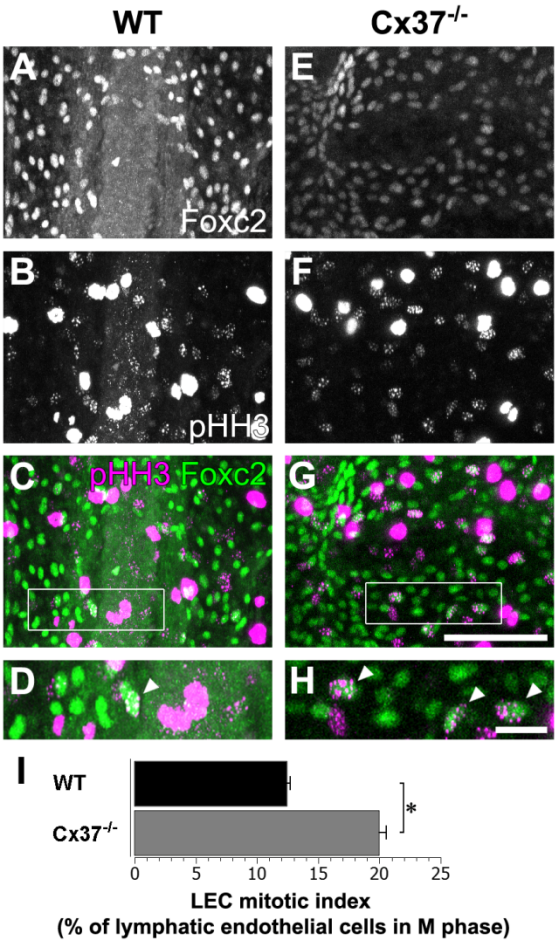


**Figure 7.** Mesenteric lymphatic endothelial cells (LECs) of Cx37-/- mice are more mitotically active than WT mice. Foxc2 (A) and phospho-Histone H3 (pHH3, B) immunolabeling of WT mesenteric lymphatic vessels. (C) Colocalization of Foxc2 (green) and pHH3 (magenta) signal highlight LECs that are in M-phase of the cell cycle. (D) Higher magnification of the white box in (C), arrowhead denotes mitotic LECs (Foxc2+ and pHH3+ cells). Foxc2 (E) and pHH3 (F) immunolabeling of Cx37-/- mesenteric lymphatic vessels. (G) A greater number of mesenteric LECs show colocalization of Foxc2 (green) and pHH3 (magenta) signal in Cx37-/- mice. (H) Higher magnification of the white box in (G), arrowheads denote mitotic LECs. (I) LEC mitotic index measured as the number of Foxc2+pHH3+ cells divided by total number of Foxc2+ cells; there is a significant increase in the percentage of mitotic mesenteric LECs in Cx37-/- mice compared to WT. Note that there is non-specific labeling of the cytoplasm of macrophages and mast cells with the pHH3 antibody, seen as the saturated signal from cells in white (B,F) and magenta (C,G,D,H). Scale bars: (A-C, E-G) 100 µm, (D,H) 20 µm. Values are presented as means, with error bars indicating standard error of the mean. Asterisks, $p < 0.05$. Student's t-test (two-tailed, unequal variance) was performed for statistics.

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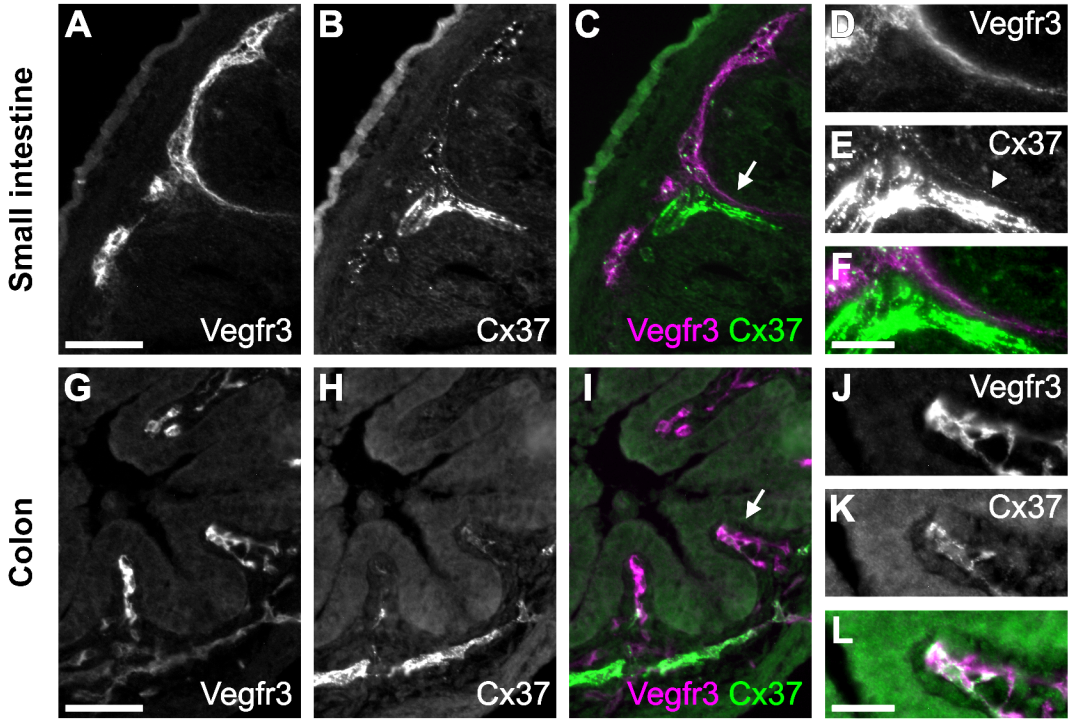


**Figure 8.** Cx37 is expressed in the submucosal lymphatics of the small intestine and colon. Cryosections of the small intestine (A-F) and colon (G-L) of E18.5 WT mice are shown. Vegfr3 immunostaining was used to identify submucosal lymphatic vessels. (A) Vegfr3 and (B) Cx37 immunolabeling of the submucosal lymphatics of the small intestine. (C) Vegfr3 (magenta) and Cx37 (green) signals colocalize in the submucosal lymphatics and lacteal. (D-F) Higher magnification view of the area denoted by the arrow in (C), arrowhead in (E) indicates relevant area of Cx37 expression within the lacteal. Vegfr3 (G) and Cx37 (H) immunolabeling of the submucosal lymphatics of the colon. (I) Vegfr3 (magenta) and Cx37 (green) signals colocalize in the submucosal lymphatics. (J-L) Higher magnification view of the area denoted by the arrow in (I). The bright, saturated Cx37 signal in (E) is from the arterial submucosal vasculature, also in the lower part of panel (H). Scale bars: (A-C, G-I) 50 µm; (D-F, J-L) 25 µm.

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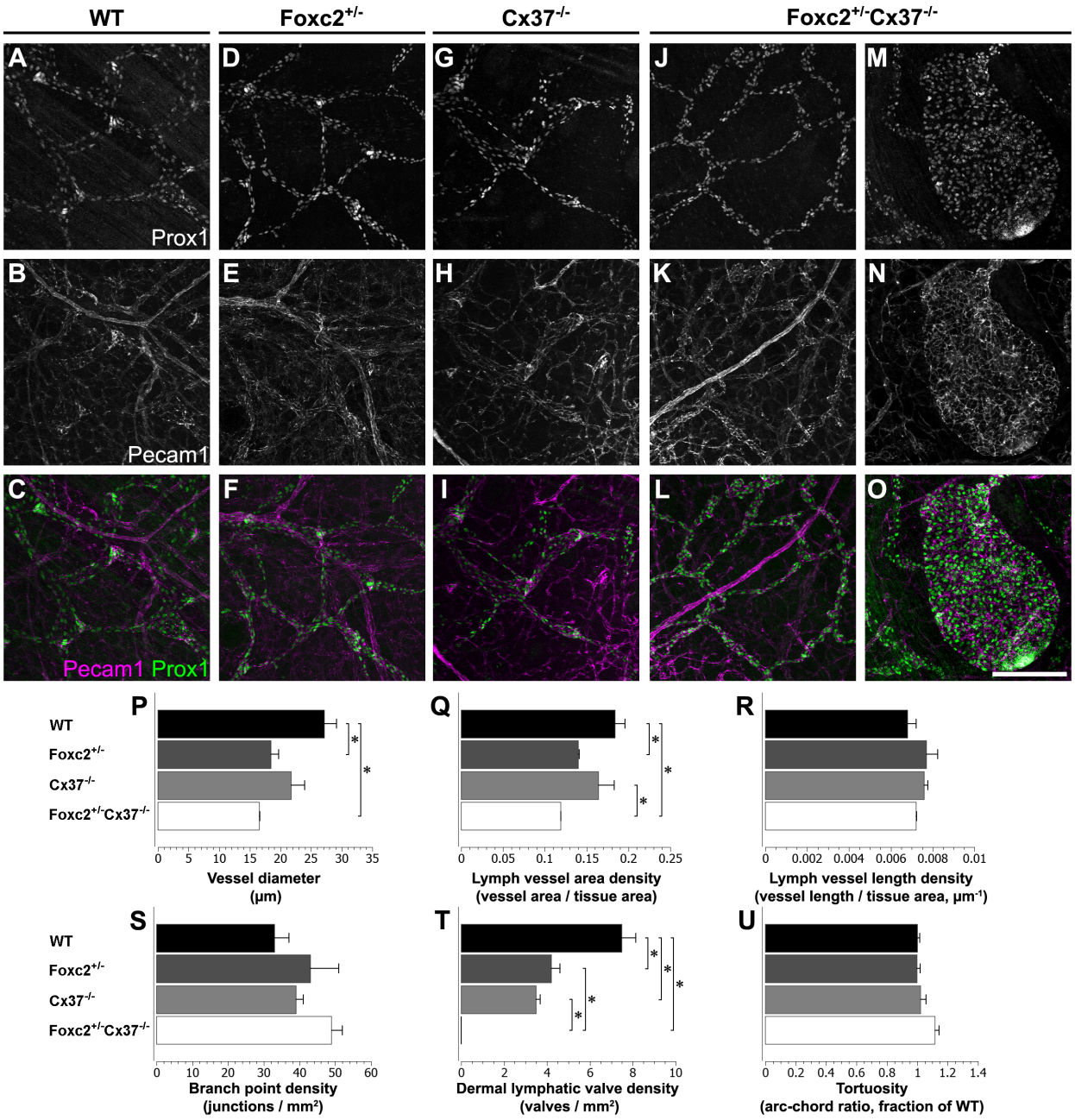


**Figure 9.** The dermal lymphatics of Foxc2+/-Cx37-/- mice at E18.5 lack valves, are smaller in caliber, and can develop structures resembling cystic lymphangiomas. Prox1 immunolabeling (A, D, G, J, M) and Pecam1 immunolabeling (B, E, H, K, N) for WT, Foxc2+/-, Cx37-/-, and Foxc2+/-Cx37-/- dermal lymphatic vessels. (C, F, I, L, O) Color composites of corresponding panels (A, D, G, J, M) for Prox1 (green) and (B, E, H, K, N) for Pecam1 (magenta). (M-O) Structure resembling a cystic lymphangioma in a Foxc2+/-Cx37-/- skin sample. (P-U) Dermal lymphatic vascular network quantitation. (P) Dermal lymph vessel diameter of Foxc2+/-Cx37-/- mice was significantly smaller than WT mice. Dermal lymph vessel diameter of Foxc2+/- mice was also significantly lower than WT. (Q) Dermal lymph vessel area density of Foxc2+/-Cx37-/- mice was significantly lower than WT and Cx37-/- mice. Additionally, lymph vessel area density of Foxc2+/- mice was significantly lower than WT. (T) There were significantly fewer dermal

lymph valves in Foxc2+/-Cx37-/- mice compared to WT, Foxc2+/-, and Cx37-/- mice. There was also a significant reduction in dermal lymph valves in Foxc2+/- and Cx37-/- mice compared to WT. There were no differences in dermal lymph vessel length density (R), branch point density (S), or vessel tortuosity (U) between genotypes. Scale bar: (A-O) 200 μm. Values are presented as means, with error bars indicating standard error of the mean. Asterisks, $p < 0.05$. Single factor ANOVA followed by post hoc Tukey-Kramer tests were performed for statistics.

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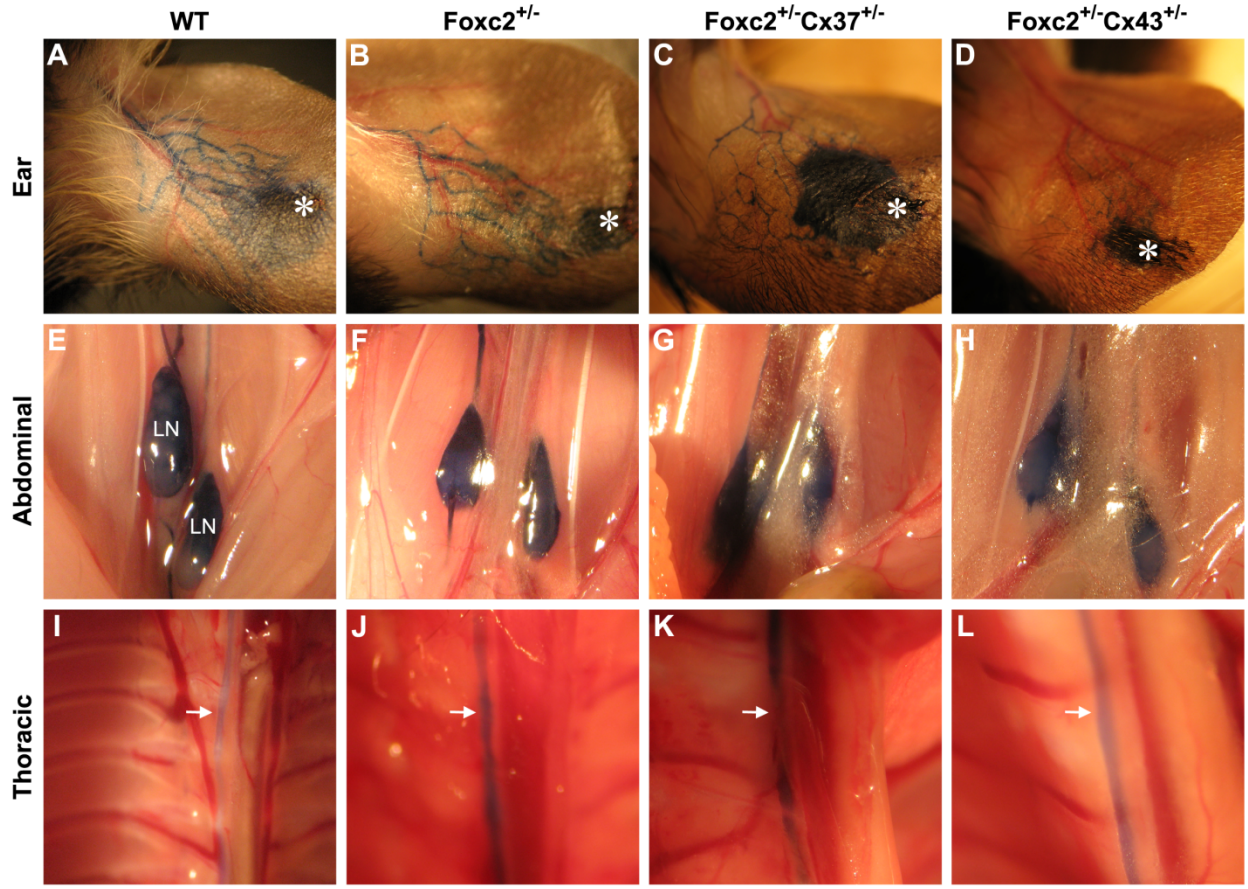


**Figure 10.** Evans blue dye (EBD) lymphangiography of WT, Foxc2+/-, Foxc2+/-Cx37+/-, and Foxc2+/-Cx43+/- adult mice. EBD drainage patterns in the ear (A-D), abdominal cavity (E-H), and thoracic cavity (I-L). EBD readily filled the lymphatics of ear (injection site denoted by the asterisk), the lumber lymph nodes (E-H, labeled "LN"), and thoracic duct (denoted by arrows in I-L) of WT, Foxc2+/-, Foxc2+/-Cx37+/-, and Foxc2+/-Cx43+/- mice. No obstructions or reflux of EBD were found in the areas examined.

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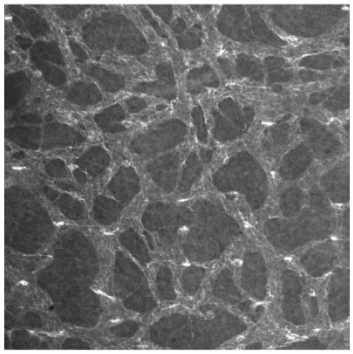
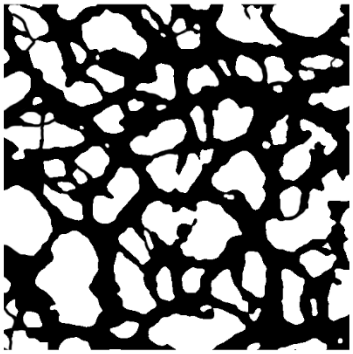
A

Original micrograph



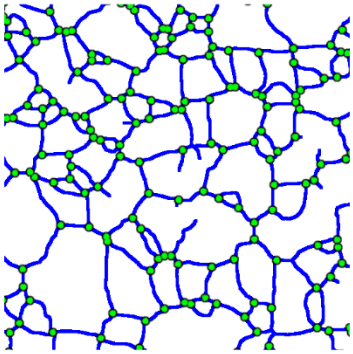
B

Segmented ly vessels



C

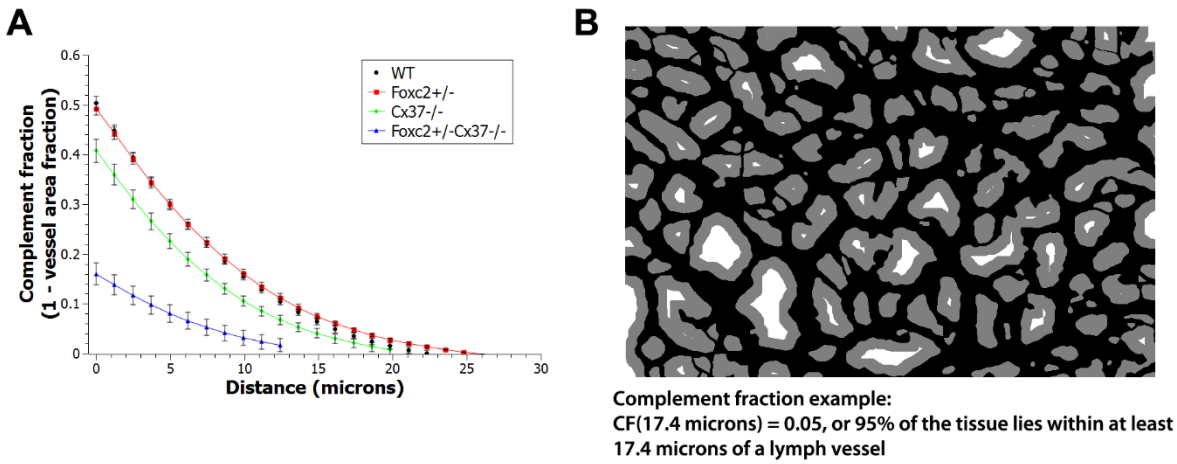
Skeletonized network



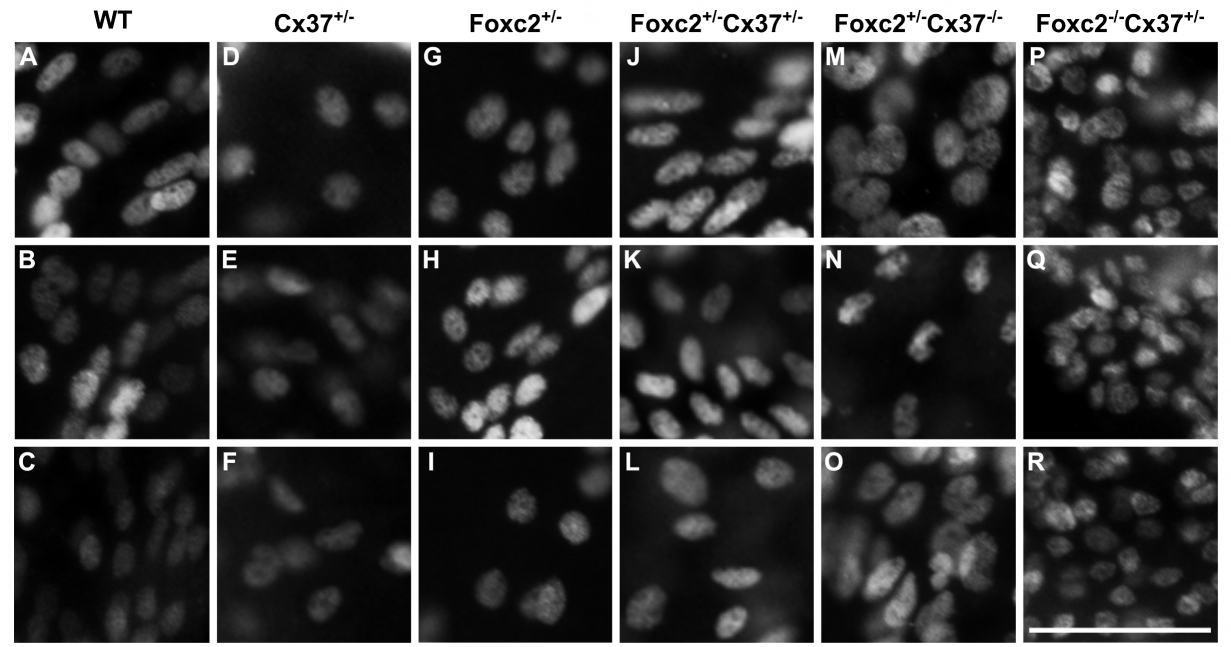
D

| Measure | Calculation | Units |
|---|---|---|
| Area density | $\frac{Total\ vascular\ area\ (mm^2)}{field\ area\ (mm^2)}$ | Dimensionless |
| Length density | $\frac{Total\ vessel\ length\ (mm)}{field\ area\ (mm^2)}$ | $mm^{-1}$ |
| Branch point density | $\frac{Number\ of\ branch\ points}{field\ area\ (mm^2)}$ | Branches / $mm^2$ |
| Mean vessel diameter | $\frac{Total\ vascular\ area\ (\mu m^2)}{total\ vessel\ length\ (\mu m)}$ | µm |
| Tortuosity | $\frac{Interbranch\ point\ vessel\ segment\ length\ (mm)}{Euclidean\ distance\ between\ branch\ points\ (mm)}$ | Dimensionless |

**Supplemental figure 1.** Vascular quantitation procedure. The original micrograph (A) was segmented (B) and a skeletonized network (C) was created using ImageJ. Green dots in the skeletonized network image highlight vessel branch points. Total vascular area was measured from the segmented image. Total vessel length, number of branch points, interbranch point vessel segment length, and Euclidean distance between branch points were measured from the skeletonized network with ImageJ. Measures, their calculation, and units are presented in table form (D).




**Supplemental figure 2.** Complement fraction of the submucosal lymphatic network for WT, Foxc2+/-, Cx37-/-, and Foxc2+/-Cx37-/- mice at E18.5. (A) Binary dilations of the segmented networks (see supplemental figure 1) were performed and complement fraction was calculated at each iteration of dilation by subtracting the lymph vessel area fraction from 1. (B) Example of complement fraction for a WT sample: black regions represent the original segmentation of the lymph vascular network, grey regions represent the 95% of the tissue that is within 17.4 microns of any given vessel.




**Supplemental figure 3.** Comparison of nucleus shape across genotypes. Prox1 immunolabeling. Each column is labeled by genotype. Three fields from the mesenteric lymphatics are shown for each genotype. Nuclei of Foxc2+/-Cx37-/- mesenteric LECs display nuclear pleomorphism and have prominent nucleoli. In some cases, nuclei are horse-shoe or kidney shaped. Scale bar: 50 µm.

# APPENDIX C: LYMPHATIC COMMUNICATION: CONNEXIN JUNCTION, WHAT'S YOUR FUNCTION?

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# Lymphatic communication: Connexin junction, what's your function?

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## Summary

This article reviews recent findings on expression and function of connexin proteins - the structural subunits of gap junction intercellular channels in the lymphatic vasculature - both during development and in the mature lymphatic vessel. Highlighted in particular are recent mouse connexin knockout studies which show that connexins are crucial for normal lymphatic development. We discuss, in general terms, both channel-dependent as well as channel-independent functions of connexins and raise some of the many unanswered questions about the mechanism(s) of action and physiological roles of connexins in the lymphatic vasculature.

### Keywords

Connexin; Gap junction; Lymphatic development; Lymphatic vasculature; Lymphatic valve

"Man is by nature a social animal," Aristotle wrote in the 4th century BC, but perhaps he did not appreciate at the time how relevant his statement would be for the microscale of our very existence. Humans are indeed social creatures. Communication is a critical part of our daily lives and occurs at various levels, from interactions with our family, friends and colleagues to larger scale community interactions (such as those within our respective fields or across fields). Additionally, the different modes in which we communicate each carry their own speeds, advantages, disadvantages, and particular functions. Much in the same way, the cells of our body communicate with each other at different levels and have diverse tools with which they carry out that communication. One of the most robust tools cells use in their interactions comes in the form of a protein family known as connexins.

Connexins are well recognized for their ability to facilitate direct cell-to-cell communication. The connexin protein family in humans comprises 21 isoforms, each with distinct functional properties, which are differentially expressed and dynamically regulated in a multitude of tissues and organs throughout the body (1,2). Six connexin proteins assemble together to form membrane channels termed hemichannels. On their own, hemichannels can act as portals that allow signaling molecules such as ATP (3), glutamate (4), and prostaglandins (5) to pass into the extracellular space (6). When docked with a hemichannel from another cell, they can directly link the cytoplasm of neighboring cells through the formation of intercellular channels, which often organize into tightly packed clusters called gap junctions. The "gap" here refers to the very close apposition of membranes at these junctions, visualized via electron microscopy as a 3 nm separation between cells. Gap junctions allow for the exchange, sometimes selectively, of a variety of substances typically less than ~1 kDa in size such as ions, metabolites, signaling molecules

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(e.g. $Ca^{2+}$, cAMP, cGMP, $IP_3$), short peptides (7), and even short nucleotide sequences in the form of miRNA (8) and siRNA (9) between cells (1). One of the most striking consequences of this cell-cell link is exemplified by the heart, where the coordinated contraction of the organ depends on gap junctions to allow the spread of ions and thus the electrical impulses that drive the muscle. Gap junctions not only mediate direct links between the same cell type (cardiomyocyte to cardiomyocyte, for instance), but also between different cell types (such as endothelial cells and vascular smooth muscle cells) (10). In addition to their importance in mediating direct cell-to-cell communication, connexins have increasingly been recognized to have channel-independent functions through their interaction with other cellular proteins (11). Connexins, through their channel-dependent and channel-independent functions, have been found to be involved in a diverse array of cellular processes including growth (12), migration (13), and differentiation (14,15) to name a few. These features of connexin function have profound consequences for the development of organisms, the coordination of normal function of tissues and organs, and the responses of cells and systems to pathophysiological situations.

While connexins have been studied extensively in the blood vasculature, the exploration of their expression and function in the lymphatic vasculature has been quite limited until recently. About three decades ago, Rhodin and Sue suggested that gap junctions might exist between lymphatic endothelial cells, but the electron micrographs were inconclusive (16). In addition, McHale and Meharg cited electron microscopy data reportedly displaying gap junctions between lymphatic smooth muscle cells (17). Gap junctions between sinus-lining endothelial cells of the rabbit lymph node were clearly visualized by electron microscopy (18) and later immunohistochemistry revealed that a specific connexin isoform, connexin43 (Cx43), was expressed by the sinus-lining endothelial cells of human lymph nodes (19). Moreover, studies of bovine and rat mesenteric lymph vessels provided evidence that synchronized and propagated contractions of lymph vessels could be impaired through the use of non-specific gap junction inhibitors (17,20). More recently, genetic linkage and DNA sequencing studies identified missense mutations in Cx47 (*GJC2*) that underlie the development of some forms of human lymphedema (21,22). Despite these important discoveries, a great deal of mystery continues to shroud connexins and their relationship with the lymphatic vasculature.

Recently, our group characterized the *in vivo* expression of three connexin isoforms – Cx37 (*Gja4*), Cx43 (*Gja1*), and Cx47 (*Gjc2*) – at various levels of the mouse lymphatic vasculature and showed that they are differentially expressed throughout embryonic and post-natal lymphatic development (23). Cx37 and Cx43 are both found during early lymphatic development in the lymphatic endothelial cells (LECs) of the jugular lymph sacs. Interestingly, their expression in the jugular lymph sac often occurs in distinct domains, with regions of high Cx37/low Cx43 expression next to regions of high Cx43/low Cx37 expression. Within developing and mature lymphatic collecting vessels, Cx37 and Cx43 colocalize in the general (non-valve) endothelium. However, during development these connexins become progressively enriched at lymphatic valves and, in mature valves, they are exquisitely differentially expressed in the upstream versus downstream sides of the valve leaflets (see the accompanying figure). Cx43 is found exclusively In the LECs of the upstream valve leaflet, whereas Cx37 is expressed exclusively in the LECs of the downstream leaflet. The significance of this segregated expression is not yet clear, but one idea is that it may represent a physiological response to unequal mechanical stress experienced by the two sides of the valve leaflet. Cx47 is also highly enriched in the endothelium of lymphatic valves and, curiously, its expression is restricted to a small subset of valve LECs, where it colocalizes with Cx43. In contrast to the abundant presence of these connexins within lymph collecting vessels, their expression in the lymphatic capillary network is very low or undetectable.

Both Cx37 and Cx43 have critical roles in lymphatic vascular development and function as evidenced by the consequences of their loss in knockout mice (23). In the early stages of lymphatic development, profound enlargement of the jugular lymph sac occurs with the loss of Cx37 alone and this effect is exacerbated when combined with the loss of Cx43. Embryos lacking both Cx37 and Cx43 also exhibit widely dilated superficial lymphatics in the skin and severe lymphedema. Amazingly, ablation of Cx43 alone results in the complete loss of valve formation in the collecting lymphatics of the mesentery. In addition, knockout of Cx43 results in an abnormally patterned thoracic duct characterized by erratic caliber, blind-ended outcroppings, and bifurcated segments. Loss of Cx37, on the other hand, results in a partial reduction in valve number in mesenteric lymphatics and adult mice that display lymphatic reflux. Combining the Cx37 knockout with the loss of a single copy of Cx43 leads to an interesting mouse model in which adult mice exhibit severe lymphatic reflux and often develop sudden lethal chylothorax – defects stemming from the deficit of lymphatic valves. In the case of chylothorax, the likely scenario is that the thoracic duct valve deficits observed in these mice encumber the capacity of the lymphatic system to the point where sudden rupture of vessels in the thoracic cavity occurs. These findings illustrate crucial functions for Cx37 and Cx43 in collecting vessel and lymphatic trunk development and function.

Given their lack of expression in lymphatic capillaries, it is perhaps not surprising that the lymphatic capillary network *per se* is seemingly unaffected by the loss of Cx37 or Cx43 (23). However, that is not to say that connexins are unimportant for lymphatic capillaries. Dicke *et al.* have recently shown that ectodermal expression of a different connexin, Cx26 (*Gjb2*), was essential for normal peripheral lymphangiogenesis in mice, as either the ablation of Cx26 or its substitution with Cx32 (*Gjb1*) led to abnormal lymphatic capillary network development and lymphedema (24). The authors propose that Cx26 may regulate lymphangiogenic signals that arise from developing epidermis, and thus influence dermal lymphatic development. In this light, it is intriguing to consider the implications of prior results documenting Cx26 expression in human breast tumors and its association with lymphatic vessel invasion (25) as well as the relationship between Cx26 expression and the likelihood of metastasis or poor prognosis in other types of tumors (26,27). From these recent studies, it is becoming more and more apparent that connexins play influential roles in the development and function of the lymphatic vasculature. Looking ahead, however, there remain many unanswered questions and a multitude of investigative avenues to venture down.

Basic questions about connexins and lymph vessels remain unanswered –namely are there *bona fide* gap junction structures in the lymphatic wall and, if so, where are they? Connexins have been studied extensively in the cardiovascular system and are known to couple blood vascular endothelial cells, vascular smooth muscle cells, as well as coupling blood vascular endothelial cells with vascular smooth muscle cells (myoendothelial coupling) (28). Whereas morphologically identifiable gap junctions have been demonstrated between the sinus-lining endothelial cells of the lymph node, gap junctions between endothelial cells of lymph vessels have not been documented. Importantly as well, functional coupling between lymphatic endothelial cells via gap junctions remains to be demonstrated. While studies employing the use of non-specific gap junction inhibitors have shed some light on a possible functional role for gap junctions in lymph vessel contraction (17,20), the non-specific inhibitors used in those studies could have had effects on other membrane proteins. Thus, studies that explore the functional coupling of cells (dye transfer or electrical coupling experiments) within lymphatic vessels are needed. It also remains unclear whether there are myoendothelial junctions between lymphatic endothelial cells and lymphatic smooth muscle. These issues are particularly pertinent in light of experiments that, through the targeted destruction of the endothelial cell layer versus the vascular smooth muscle layer of

arterioles, showed that dilatory and vasoconstrictive signals could be differentially blocked by the destruction of one cell layer versus the other (29). Clearly, elucidating the communicating partners within and between layers of the lymphatic vessel wall will be critical to enhancing our understanding of the cellular interplay that coordinates lymph vessel contraction, relaxation, and propagated vascular responses.

The previous questions inevitably lead to the following – what are connexins and/or gap junctions actually doing in the lymphatic system? In considering this, insight may be gleaned from their functions in other cells and systems. Their possible role in mediating lymph vessel contraction has been alluded to previously and likely involves their ability to facilitate the spread of depolarizing or hyperpolarizing currents through the vessel wall. However, gap junctions in lymphatics may also mediate the transfer of other permeants such as second messengers (e.g. $Ca^{2+}$, cAMP, cGMP, $IP_3$) or something akin to endothelium-derived hyperpolarizing factor, as has been hypothesized in the blood vasculature (30). But, this assumes gap junctions are indeed coupling cells within lymph vessels. In addition (or perhaps, alternatively), connexins could be forming hemichannels, and their ability to allow substances to be released into the surrounding tissues or lumen of the vessel, perhaps in response to stretching of the vessel wall, could theoretically affect lymph vessel dynamics through paracrine signaling (31).

In terms of development, the potential influences of connexins on the lymphatic vasculature are numerous. Cx37 and Cx43 have both been shown to mediate growth control effects in *in vitro* settings (32,33). Hence, these connexins may be exerting sway over lymphangiogenesis through modulation of proliferation. Connexins have also been implicated in developmental patterning (34–36) and in the control of cell migration (37). Studies in the blood vasculature suggest that the precise level of connexin expression can affect vascular network formation, as the loss of a single copy of Cx43 in mice resulted in variable branching and abnormal patterning of coronary vessels (38). We have demonstrated the profound impact on collecting vessel formation that occurs with the loss of Cx37 and Cx43, particularly for valve development, but these connexins may also be involved in the patterning of the lymphatic network, as suggested by the abnormal patterning of the thoracic duct and intercostal lymphatic trunks in Cx43 knockout mice (23). However, connexin involvement in the development of vascular beds (blood or lymph) remains poorly understood. In both these regards (growth and development), connexins represent very attractive targets of study for their possible involvement in directing and modulating physiological and pathophysiological lymphangiogenesis.

An important challenge ahead will be to understand how connexins are integrated with other signaling pathways and genetic programs during lymphatic development, particularly because it is still uncertain whether their effects are mediated through their channel functions or through their ability to potentially interact with a host of intracellular proteins such as scaffolding proteins, cytoskeletal elements, kinases, and, in the case of Cx37, endothelial nitric oxide synthase (39). It is beyond the scope of this article to review in depth the substantial progress that has been made in recent years in identifying crucial genes and proteins involved in lymphvasculogenesis and lymphangiogenesis. A number of approaches have established the importance of key transcription factors, signaling proteins, receptors, and cell-matrix interactions in the developmental sequence (40). One of the transcription factors found to be important for lymphatic collecting vessel development is Foxc2, a forkhead family transcription factor that is mutated in human lymphedema-distichiasis syndrome and is required for lymphatic valvulogenesis (41–43). Interestingly, Cx37 expression is drastically reduced in the jugular lymph sac and mesenteric collecting vessels of mice that lack Foxc2, suggesting that Cx37 may be a target of regulation by Foxc2 (23). Besides Foxc2, a number of other proteins have been shown to be critical for lymphatic

collecting vessel development and valve formation, including Integrin-α9 (44), EphrinB2 (45), and NFATc1 (46), and it will be interesting to see if connexins in some way contribute to these signaling pathways in lymphatic development.

The exploration of connexins in the lymphatic system represents fertile scientific territory. Some of the groundwork has been laid in the last 30 years, but there are still many unanswered questions. The importance of connexins in facilitating the vascular tone of blood vessels is well recognized, yet knowledge of their function in the contractile activity of lymph vessels is minimal. The developmental roles of connexins are diverse, but we are still only scratching the surface in terms of figuring out their precise functions and mechanisms of action both in non-lymphatic and lymphatic systems. If we are to understand the lymph vasculature more completely, we will need to appreciate more clearly how the cells of the system communicate with each other. And, while cells may not communicate with words, the better our understanding of their language and the ways in which that communication affects their behavior, the shorter our stay will be in the shadow of ignorance.

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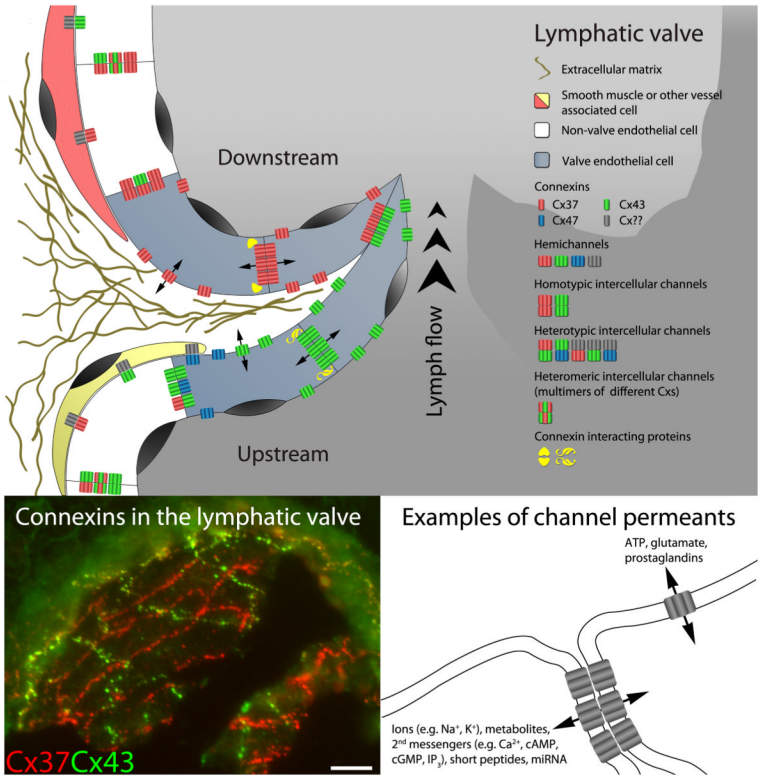


**Figure.**

*Top*. Schematic representation of a longitudinal section through a lymphatic collecting vessel with emphasis on a valve. Depicted are potential connexin-containing channel configurations that may exist on or between cells of the vessel wall. Channel types: hemichannel (six connexin subunits assembled into a channel in one membrane), homotypic (composed of matching hemichannels), heterotypic (composed of non-matching hemichannels), and heteromeric (composed of hemichannels containing more than one connexin type). Proposed channel combinations are not exhaustive.

*Bottom left*. Cx37 (red) and Cx43 (green) immunofluorescent staining of a transverse section through a mesenteric lymph valve (mouse, 4 weeks old). The valve leaflet shown appears *en face* in the section and extends into the lumen of the vessel. Cx37 and Cx43 are highly enriched in the valve and are differentially expressed on the two sides of the valve leaflet. Cx43 is present on the upstream side of the valve leaflet and Cx37 is present on the downstream side. Colors correspond with connexin colors in the accompanying schematic. Scale = 10 μm. This image is reprinted from Kanady *et al*. (23) with permission from Elsevier.

*Bottom right*. Examples of hemichannel and gap junction intercellular channel permeants as documented in other cell types.