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The elephant in the cell

A multifaceted undertaking to uncover the molecular principles behind lymphatic endothelial cell identity

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Abstract

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The lymphatic vascular network is composed of a series of blind ended vessels, and is involved in physiological functions such as fluid homeostasis, lipid metabolism and immune trafficking.

Despite lymphatic endothelial cells being derived from multiple organs, they share common molecular denominators, including the expression of the transcription factor Prox1. However, many of the molecular mechanisms involved in the acquisition and maintenance of lymphatic identity remains to be uncovered.

The aim of my thesis is to investigate how transcription factors and chromatin re-arrangements are involved in the acquisition and maintenance of lymphatic identity. In Paper I, the role of the transcription factor mafbb and its ability to partially compensate for the loss of its paralog mafba is investigated, leading to the description of topologically distinct requitements for LECs development in zebrafish. In Paper II, an evolutionary conserved prox1a enhancer is shown to be necessary for correct lymphangiogenesis in mouse, and its ablation is shown to cause the transition of LECs to hematopoietic-like cells. In Paper III, the effects of partial and total prox1 loss in zebrafish are investigated from a transcriptomic and epigenetic perspective, revealing an important role for prox1 in establishing LEC identity by suppressing genes associated with VEC fate. In Paper IV, the cis-regulatory landscape of prox1a is characterised identifying a number of lymphatic enhancers driving expression in either all LECs in the embryo or in anatomically distinct subsets of lymphatic vasculature. Finally, in Paper V, the chromatin organisation signature of LECs is explored, finding new candidate genes and enhancers active in the lymphatic endothelium.

In summary, my doctoral studies investigated the different levels of regulation of cell identity involved in LECs differentiation. My work focused on chromatin organisation, enhancer activity, transcription factors and differential gene expression to uncover the complex interactions between these separate mechanisms.

Keywords: Lymphatic endothelium, Development, Epigenetic

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To Germana, who couldn't be here.

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I. Arnold H, **Panara V**, Filipek-Gorniok B, Skoczylas R, Ranefall P, Gloger M, Hogan BM, and Koltowska K. (2022) 'Mafba and Mafbb Differentially Regulate Lymphatic Endothelial Cell Migration in Topographically Distinct Manners'. *Cell Reports*, 39(12):110982.

II. Kazenwadel J, Venugopal P, Piltz S, Brown C, Taoudi S, Thomas P, Scott H, Oszmiana A, Toubia J, Arriola L, **Panara V**, Koltowska K, Ma W, Schreiber A, Harvey N. (2023) 'Lymphatic endothelial cell identity is controlled by a GATA2 bound enhancer'. *Nature* 614, 343–348.

III. Grimm L, Mason E, Yu O, Dudczig S, **Panara V**, Chen T, Bower NI, Paterson S, Okuda K, Rondon Galeano M, Kobayashi S, Senabouth A, Lagendijk AK, Powell J, Smith KA, Koltowska K, Hogan BM. (2023) 'Single cell analysis of lymphatic endothelial cell fate specification and differentiation during zebrafish development'. *EMBO Journal* e112590.

IV. **Panara V**, Yu O, Peng D, Mason E, Skoczylas R, Allalou A, Staxäng K, Hodik M, Haitina T, Hogan BM, Koltowska K. 'Distinct *cis*-regulatory elements drive *prox1a* expression in specific lymphatic vascular beds'. ***Manuscript***

V. **Panara V*,** Arnold H*, Gloger M*, Smialowska A, Johansson A, Skoczylas R, Filipek-Gorniok B, Koltowska K. 'Multiomic approach reveals different chromatin signature in lymphatic and blood endothelial cells'. ***Manuscript***

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Additional publications

I. **Panara V**, Monteiro R, Koltowska K. (2022) 'Epigenetic Regulation of Endothelial Cell Lineages During Zebrafish Development - New Insights from Technical Advances'. *Front. Cell Dev. Biol.* 10.

Contents

Abbreviations

ADAMTS2	ADAM metallopeptidase with Thrombospondin type 1 motif 2
ADAMTS3	ADAM metallopeptidase with Thrombospondin type 1 motif 3
ADAMTS14	ADAM metallopeptidase with Thrombospondin type 1 motif 14
AKT	AKT serine/threonine kinase 1
ATAC-seq	Assay for Transposase-Accessible Chromatin with high-throughput sequencing
BEC	Blood Endothelial Cell
Cas9	Type II CRISPR RNA-guided endonuclease Cas9
CCBE1	Collagen and Calcium Binding EGF domains 1
CCV	Common Cardinal Vein
cdh5	Cadherin 5
cdh6	Cadherin 6
ChIP-seq	Chromatin Immunoprecipitation sequencing
cldn11a	*Claudin 11a*
COUP-TFII	COUP Transcription Factor 2
CRE	*Cis*-Regulatory Element
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
CTCF	CCCTC-binding Factor
CUT&RUN	Cleavage Under Targets & Release Using Nuclease
CUT&Tag	Cleavage Under Targets & Tagmentation
CV	Cardinal Vein
cxcl12a	Chemokine (C-X-C motif) Ligand 12a
DA	Dorsal Aorta
DLAV	Dorsal Longitudinal Anastomosing Vessel
dll4	Delta Like canonical Notch Ligand 4
DLLV	Dorsal Longitudinal Lymphatic Vessel
dpf	Days Post Fertilisation
EC	Endothelial Cell
ERK	Extracellular Regulated MAP Kinase
Etsrp	ETS1-Related Protein
FAC	Fluorescence-activated cell (sorting)
FCLV	Facial Collecting Lymphatic Vessel
fli1b	Fli-1 proto-oncogene ETS transcription factor b
flt1	FMS-like Tyrosine kinase 1

flt4	FMS-like Tyrosine kinase 4
foxc1a	Forkhead box C1A
Foxc1	Forkhead box C2
Gata2	GATA binding protein 2
gata2a	GATA binding protein 2a
H3K4me1	Monomethylation of the lysine 4 of histone 3
H3K4me3	Trimethylation of the lysine 4 of histone 3
H3K27ac	Acetylation of lysine 27 of histone 3
Hi-C	Chromosome Conformation Capture
HM	Horizontal Myoseptum
hpf	Hours Post Fertilisation
hlx1	H2.0-like homeo box 1
ISLV	Intersegmental Lymphatic Vessel
Kdmr4a	Lysine demethylase 4A
Kdmr4c	Lysine demethylase 4C
kdr	Kinase insert Domain Receptor
kdrl	Kinase insert Domain Receptor-Like
KLF4	Kruppel-like factor 4
LAA	Lymphatic branchial arches
LEC	Lymphatic Endothelial Cell
LFL	Latero-Facial Lymphatic
Mafb	v-Maf Avian musculoaponeurotic Fibrosarcoma oncogene homolog B
mafba	v-Maf Avian musculoaponeurotic Fibrosarcoma oncogene homolog Ba
mafbb	v-Maf Avian musculoaponeurotic Fibrosarcoma oncogene homolog Bb
MFL	Medio-Facial Lymphatic
MZ	Maternal Zygotic
nfatc1	Nuclear Factor of Activated T Cells 1
notch1b	Notch receptor 1b
nrp1b	Neuropilin 1b
OLV	Otolithic Lymphatic Vessel
PCV	Posterior Cardinal Vein
Prdm16	PR Domain containing 16
PHS	Primary Head Sinus
PLs	Parachordal Lymphangioblasts
Prmt5	Protein arginine Methyl Transferase 5
Prox1	Prospero-Related homeobox 1
prox1a	Prospero-Related homeobox 1a
prox1b	Prospero-Related homeobox 1b
RUNX1	RUNX family transcription factor 1
scRNA-seq	Single-cell RNA sequencing
sox7	SRY-box transcription factor 7

sox18	SRY-box transcription factor 18
TAD	Topologically Associated Domain
tbx1	T-Box 1
TF	Transcription Factor
TSS	Transcription Start Site
VA-L	Ventral Aorta Lymphangioblast
VEC	Venous Endothelial Cell
vegfa	Vascular Endothelial Growth Factor A
vegfc	Vascular Endothelial Growth Factor C
vegfd	Vascular Endothelial Growth Factor D
VEGFR2	Vegf Receptor 2
VEGFR3	Vegf Receptor 3
vISV	Venous Intersegmental Vessel
WT	Wild Type

Populärvetenskaplig sammanfattning

Hjärt- och kärlsystemet är fundamentalt när det kommer till att transportera syre och essentiella näringsämnen i kroppen. Systemet innefattar hjärtat som pumpar blodet, artärerna som för syre och näringsrikt blod till kroppens olika delar, venerna som för tillbaka näringsfattigt blod till hjärtat, samt kapillärerna. Dessa är tunna kärl som sitter mellan artärer och vener. I kapillärerna kan den flytande delen av blodet ta sig ur kärlen och utbyta näringsämnen med kroppens celler. En del av denna vätska kan återigen absorberas av venerna, men en stor del tas istället upp av ett annat, specialiserat kärlsystem: lymfkärlen.

Lymfkärlen tillsammans med andra organ, till exempel lymfknutor, benmärgen och mjälten utgör lymfsystemet. Detta är viktigt när det kommer till immunfunktion, absorption av lipider och till att reglera vätskemängden i vävnader. Via ärftliga åkommor som påverkar detta system, eller till följd av operation (till exempel att lymfnoder tagits bort som en del i behandlingen av bröstcancer) kan man råka ut för något som heter lymfödem. Det mest märkbara symtomet vid lymfödem är uppsvullna kroppsdelar där lymfan, vätskan i lymfsystemet, ansamlats och inte kan föras bort ifrån. I nuläget finns inget botemedel mot lymfödem och därför är det viktigt att undersöka de mekanismer som är involverade i framväxten av lymfsystemet så att potentiella behandlingsvägar kan identifieras.

Under embryots utvecklingsfas kommer de celler som ska komma att utgöra lymfkärlen från kroppens vener. Dessa celler migrerar från dessa vener och förökar sig för att bilda kroppens huvudsakliga lymfkärl. Samtidigt måste dessa celler ändra sin identitet från ven- till lymfatiska endotelceller, alltså de celler som tillsammans bygger upp det innersta lagret i lymfkärlen, det som är i kontakt med den dränerade lymfan. För att göra det omorganiserar de strukturen på sitt DNA så att vissa gener som är typiska för blodkärlens endotelceller stängs av, medan andra som är typiska för lymfkärl sätts igång.

Under mina doktorandstudier har jag studerat just hur de lymfatiska endotelcellerna får sina karakteristiska drag. Som modellorganism har jag använt zebrafisk. Först undersökte vi vilken roll en gen som heter *mafbb* har i lymfkärlens utveckling. *mafbb* behövs normalt inte under denna process, men om en gen som är nära släkt, *mafba*, inte fungerar som den ska, så kan *mafbb* kompensera för detta och se till att lymfkärlen i ansiktet utvecklas som de ska, dock inte för lymfkärlen utanför huvudet. Detta tyder på att lymfkärlen inte är av en enhetlig “typ” utan har olika karaktärsdrag i olika delar av kroppen.

I den andra delen av mina doktorandstudier undersökte jag hur de lymfatiska endotelcellerna “bestämmer sig” för att uttrycka *prox1a*, en av de viktigaste generna för lymfkärlens utveckling. Jag identifierade ett antal områden som omger *prox1a*-genen i DNA:t. Dessa reglerar i vilka celler och vid vilken tidpunkt genen slås på, bland annat i delar av lymfkärlen. Det är av yttersta vikt att undersöka sådana här områden eftersom de kan vara inblandade i ärftliga sjukdomar. Detta genom att slå på och av genen och på så vis proteinproduktionen i fel celler, snarare än genom att producera ett felaktigt protein.

Slutligen, i den sista delen av doktorandstudierna fokuserade jag på hur DNA:ts 3D-struktur förändras när de endotelcellerna byter från ven- till lymfidentitet. Den här studien pågår fortfarande. Hittills har jag använt olika tekniker för att jämföra likheter och skillnader i DNA:ts 3D-organisation mellan blod- och lymfceller. Med detta identifierade jag flera DNA-sekvenser som är aktiva i lymfceller.

Tillsammans utgör resultaten från dessa studier en viktig grund för framtida forskning om hur skillnader DNA-organisation är kopplade till lymfsjukdomar.

The lymphatic vascular system

The lymphatic vascular network is composed of a series of blind ended vessels present in most of the organs in the vertebrate body, and it has an important function in the absorption of the fluid that has drained into the interstitial space from the capillary bed. This interstitial fluid, also called lymph, enters the lymphatic network at the level of the lymphatic capillaries and is transported through the collecting vessels to the subclavian vein, where it re-enters blood circulation through the lympho-venous valve. In addition to this important function in fluid homeostasis, the lymphatic vascular network has a role in lipid absorption and immune trafficking.

In humans, the main pathology associated with the lymphatic vasculature is lymphoedema, which manifests as a swelling of the limbs as a consequence of lymph drainage failure. Lymphoedema can develop as a consequence of intrinsic defects in the vessel formation (primary lymphoedema), or after a traumatic injury to the lymphatic network, for example after lymph node removal in breast cancer surgery (secondary lymphoedema) (Finnane *et al.*, 2015). As of today, there is no cure for lymphoedema, and treatment options focus on alleviating the symptoms and preventing the progression of the disease, often resorting to mechanical compression of the affected limb through bandaging or surgery. Lymphoedema can have a serious impact on the quality of life of the patients, so the identification of factors involved in lymphatic vascular development as possible therapeutic targets for treating the disease is of the utmost importance.

Compared with other vascular structures, the lymphatic vascular network was discovered late in the history of medicine. This is due to its anatomical and histological properties: unlike blood, the lymph is mostly transparent, and the walls of lymphatic vessels are thinner than their venous or arterial counterparts. It was only in 1653 C.E. that the vascular network was described by Olaus Rudbeck and Thomas Bartholin, independently. For comparison, the first descriptions of blood vasculature appear in ancient Egyptian medical texts, and the presence of two different types of vessels, veins and arteries, was noted by Galen in the II century C.E., more than a thousand years before the first anatomical description of a non-lacteal lymphatic vessel. After its discovery in humans, the lymphatic vasculature was in the following centuries described in other vertebrates, including teleost fishes (Hewson, 1769). However, the homology between the fish lymphatic network and the human one has been a controversial topic (see Hedrick *et al*., 2013 for a review of the historical debate), until the discovery of a lymphatic vasculature in zebrafish (Küchler *et al*., 2006; Yaniv *et al*., 2006). These foundational studies in fish lymphatics, which would grow in number in the following years, showed the developmental and molecular similarities between mammalian and teleost lymphatics, demonstrating their common evolutionary origin and a homologous function.

Lymphatic vascular network formation in zebrafish

With the discovery of a lymphatic vascular network in zebrafish, this animal has become a central model for investigating lymphatic development, due to the ease of genetic manipulation and the many molecular tools available. Lymphatic vessel development starts during the second day of zebrafish development, after the arterial angiogenic process is completed, and by 5 days post fertilisation (dpf) the main lymphatic vessels have formed.

The embryonic zebrafish lymphatic network includes facial lymphatic, trunk lymphatic, intestinal or gut lymphatics and lateral lymphatics (Okuda *et al*., 2012). At later stages the collateral cardinal lymphatics, the deep spinal lymphatics, the superficial lateral lymphatics and the superficial intersegmental lymphatics are formed (Jung *et al*., 2017). Of these subsets of vessels, the trunk and facial lymphatics have been the most extensively studied and documented in the literature.

The trunk lymphatics are composed of a ventral axial vessel, the thoracic duct (TD), which is located between the posterior cardinal vein (PCV) and the dorsal aorta (DA) (**Figure 1**). The intersegmental lymphatic vessels (ISLVs) which connect the TD and the dorso-longitudinal lymphatic vessel (DLLV), positioned ventrally to the dorsal longitudinal anastomosing vessel (DLAV) (Hogan *et al*., 2009a; Küchler *et al*., 2006; Yaniv *et al*., 2006) (**Figure 1**).

In the trunk, the acquisition of lymphatic endothelial cell (LEC) fate starts around 24 hours post fertilisation (hpf), when a population of endothelial cells (ECs) in the ventral PCV proliferates and migrates to the dorsal part (Nicenboim *et al*., 2015). Here, bipotential *Prospero-related homeobox 1a* (*prox1a*) positive progenitors can be observed at around 30 hpf (Koltowska *et al*., 2015a). At around 36 hpf, sprouts emerge from the PCV in correspondence of the pre-existing arterial intersegmental vessels (aISVs), and form transient three-way connections between the PCV, the DA and the aISV. In around half of the cases, the connection with the PCV is stabilised and the sprouts anastomise with the arteries, forming the mature venous intersegmental vessels (vISVs). In the other half, the connection between the sprout and the aISV regresses. These sprouts lose contact with the PCV and sprout dorso-laterally along the aISVs, reaching the horizontal myoseptum (HM) (**Figure 1**) (Bussmann *et al*., 2010; Geudens *et al*., 2019). While in this location, the population of LECs precursors takes the name of parachordal lymphangioblasts (PLs). PLs proliferate in the HM and then start migrating both dorsally and ventrally at approximately 60 hpf to form the mature trunk vasculature at around 5 dpf (**Figure 1**) (Hogan *et al*., 2009a; Koltowska *et al*., 2015b). This process shows remarkable similarities to mice lymphangiogenesis, where PROX1 positive cells can be observed in the cardinal vein (CV) (Wigle and Oliver, 1999). These cells then migrate dorsally to form transient structures, the lymph sacs, which are then re-modelled into a functional vasculature (Wigle and Oliver, 1999).

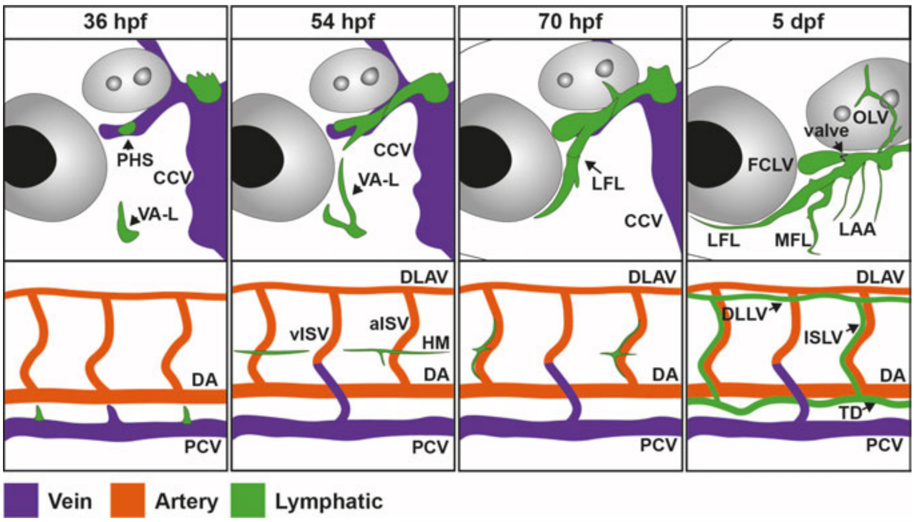

Figure 1: Schematics of lymphatic development in the head and trunk of zebrafish. At 36 hpf, *prox1a* positive progenitors (green) sprout from the PCV and the CCV. At 54 hpf, the progenitors are located at the HM in the trunk. In the facial lymphatics, the progenitors from the PHS have fused with the lymphatic sprout. At 70 hpf, migration has begun in the trunk, while the VA-L derived progenitors fuse with the developing LFL. At 5 dpf, the main vessels of both trunk and facial lymphatics are present. aISV: arterial intersegmental vessel, CCV: common cardinal vein, DA: dorsal aorta, DLAV: dorsal longitudinal anastomotic vessel, DLLV: dorso-longitudinal lymphatic vessel, HM: horizontal myoseptum, ISLV: intersegmental lymphatic vessel, LAA: lymphatic branchial arches, LFL: latero-facial lymphatic, MFL: medio-facial lymphatic, OLV: otolithic lymphatic vessel, PHS: primary head sinus, TD: thoracic duct, VA-L: ventral aorta lymphangioblast, vISV: venous intersegmental vessel. Adapted from Eng *et al*. (2019), Koltowska *et al*. (2013), Okuda *et al*. (2012).

Contrary to the highly modular structure of the trunk, facial lymphatics form a network of distinct vessels reaching the whole surface of the head. At 5 dpf, five subsets of facial lymphatics have been described: the latero-facial lymphatic (LFL), which runs ventral to the eye; the otolithic lymphatic vessel (OLV), close to the ear; the medio-facial lymphatic (MFL), branching from the LFL; and the lymphatic branchial arches (LAA) (Okuda *et al*., 2012)

(**Figure 1**). The upper-branching portion of the facial lymphatics is called the facial collecting lymphatic vessel (FCLV) (**Figure 1**), and it connects to the primary head sinus (PHS) through the lympho-venous valve (Shin *et al*., 2019).

The facial lymphatics have three different cellular origins: the common cardinal vein (CCV), the PHS and the ventral aorta lymphangioblast (VA-L). The latter is the only non-venous lymphatic endothelial progenitor population that has been described in zebrafish so far (Eng *et al*., 2019). Cells start sprouting anterior-ventrally from the CVV at 36 hpf. At 48 hpf, they merge with the PHS-derived population, and join the VA-L derived cells at around 72 hpf, maturing into the complete facial lymphatics before 5 dpf (Eng *et al*., 2019; Okuda *et al*., 2012) (**Figure 1**).

The lymphatic valve is a recently described feature of the zebrafish lymphatic system. The valve starts developing at the junction between the LFL and the FCLV at 4 dpf (Shin *et al*., 2019) (**Figure 1**), and it is marked by the expression of *gata2a* (Shin *et al*., 2019).

Factors regulating lymphatic vascular development

The development of lymphatic vessels is a complex process, involving the regulation of lineage specification, cell proliferation, sprouting and migration in order to produce a functional lymphatic vascular network. Since the first description of the zebrafish lymphatics in 2006 (Küchler *et al*., 2006; Yaniv *et al*., 2006), many of the genes contributing to lymphatic development have been characterised. Despite some key divergences, their orthologs often play a comparable role in mammalian lymphatic development. Although our understanding of several key components in lymphatic vessel development remains incomplete, we can draw a general picture of the main signalling pathways and transcription factors regulating lymphangiogenesis across Osteichthyes.

VEGFC/FLT4 is a key signalling axis for lymphatic vessel development

Vascular endothelial growth factor C (VEGFC) is essential in regulating key LEC progenitor cellular processes (Karkkainen *et al*., 2004; Küchler *et al*., 2006; Yaniv *et al*., 2006). VEGFC is a secreted signalling molecule (Joukov *et al*., 1996), and therefore much of its regulation happens in the extracellular environment (Jeltsch *et al*., 2014). Collagen and calcium binding EGF domains 1 (CCBE1) is a secreted protein which aids in cleaving VEGFC into its active form (Le Guen *et al*., 2014; Hogan *et al*., 2009a; Jeltsch *et al*., 2014). Mutations of *CCBE1* in humans cause Hennekan syndrome, leading to congenital lymphatic malformations (Alders *et al*., 2009). In both zebrafish (Hogan *et al*., 2009a) and mouse (Bos *et al*., 2011), the loss of *CCBE1* causes a complete loss of the lymphatic vascular network.

In mouse, CCBE1 has been shown to bind the ADAM metallopeptidase with thrombospondin type 1 motif 3 (ADAMTS3), a secreted protein with proteolytic activity, and both of these factors are needed for the production of mature VEGFC (Jeltsch *et al*., 2014). *Adamts3* mutant mice phenocopy *Vegfc* mutants (Janssen *et al*., 2016), further confirming the central role of extracellular processing of VEGFC for lymphangiogenesis. In humans, mutations of ADAMTS3 cause Hennekan syndrome (Brouillard *et al*., 2017), as seen for

CCBE1 mutations. In adult mice ADAMTS3 is not expressed and its functions are performed by ADAMTS2 and ADAMTS14 (Dupont *et al*., 2022). In zebrafish two orthologs, Adamts3 and Adamts14, interact with Ccbe1 in a compensatory manner (Wang *et al*., 2020).

The vascular endothelial growth factor receptor 3 (VEGFR3, encoded by the *FLT4* gene) is the main receptor for VEGFC signalling, and it is positioned upstream of intracellular AKT and ERK signalling. However, VEGFC also has the ability of binding VEGFR2 (also known as Kdr), while VEGFR3 can interact with VEGFD in mammals. In both mouse and zebrafish, VEGFC signalling controls LECs sprouting out of the PCV. Loss of either VEGFC (Hägerling *et al*., 2013; Hogan *et al*., 2009b; Karkkainen *et al*., 2004; Küchler *et al*., 2006; Yaniv *et al*., 2006) or VEGFR3 (Hägerling *et al*., 2013; Hogan *et al*., 2009b; Shin *et al*., 2016) results in serious lymphatic impairments in both species. Mutations of *FLT4* and *VEGFC* in humans are respectively the cause of Milroy disease (Karkkainen *et al*., 2000; Mendola *et al*., 2013) and Milroy disease-like phenotype (Gordon *et al*., 2013), characterised by congenital bilateral limb lymphoedema.

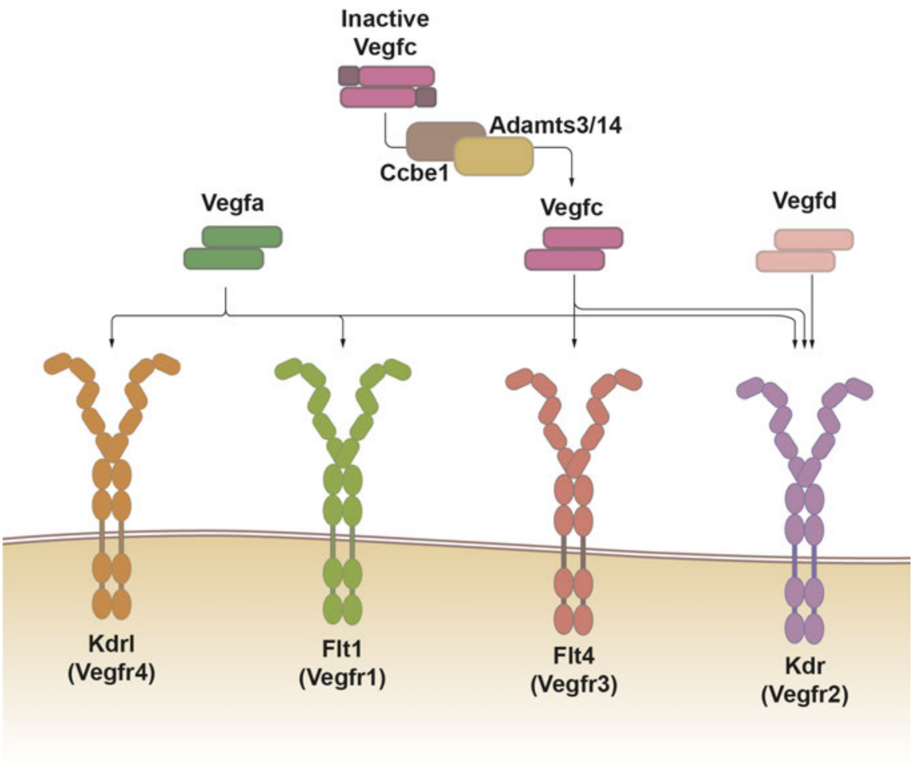

Figure 2: Schematic representation of Vegf signalling in zebrafish vascular development. Adamts3/14 and Ccbe1 mediate the processing of secreted inactive Vegfc into its active form, able to bind to Flt4 (Vegfr3) and Kdr (Vegfr2). Vegfd signals exclusively through Kdr during lymphatic development, while Vegfa, important for blood vessel formation, can signal through Flt1 (Vegfr1), Kdr (Vegfr2) and Kdrl (Vegfr4), an ohnolog of Vegfr absent in placental mammals. Adapted from Vogrin *et al*., 2019.

In zebrafish, the impairment of the VEGFC signalling pathway leads to a severe reduction of secondary angiogenesis, as well as the complete absence of trunk lymphatics (Le Guen *et al*., 2014; Hogan *et al*., 2009a). The development of the facial lymphatics depends on this pathway as well, and it is compromised upon *flt4* knock-down (Okuda *et al*., 2012). However, in *vegfc* morphants and mutants the facial lymphatics are still present, albeit reduced (Astin *et al*., 2014), and the same facial phenotype is caused by the inactivation of ERK signalling (Shin *et al*., 2016). It has been demonstrated that this weaker phenotype observed in *vegfc* mutants is caused by the compensatory effect of *vegfd*, and indeed facial lymphatics fail to develop if both *vegfc* and *vegfd* are lost (Astin *et al*., 2014; Vogrin *et al*., 2019). Moreover, the reduction of LFL and MFL in *vegfd* mutants suggests that *vegfd* does not simply compensate for the loss of *vegfc*, but has a constitutive role in the development of facial lymphatics (Bower *et al*., 2017). Further studies have determined that the compensatory mechanism is mediated by Vegfd signalling through Kdr, and not Flt4, which is not able to bind zebrafish Vegfd (Vogrin *et al*., 2019) (**Figure 2**). This compensatory mechanism is topologically restricted to the face, since *vegfd* deletion does not cause any additional trunk lymphatics phenotype (Astin *et al*., 2014; Bower *et al*., 2017), suggesting that lymphatic development could involve slightly different developmental programs in the face and the trunk.

Upon further dissection of the distinct functions of VEGFC in lymphatic development, a role in specification, sprouting, proliferation and migration has been revealed. In zebrafish, Vegfc has a function in LEC progenitor specification by inducing *prox1a* expression while still in the PCV (Koltowska *et al*., 2015a). In mouse, VEGFC depletion does not interfere with the initial specification of LECs, which still express *Prox1* even in VEGFC absence (Karkkainen *et al*., 2004). However, VEGFC is required for the maintenance of lymphatic identity (Johnson *et al*., 2008).

VEGFC signalling also controls the sprouting of LECs from the PCV. In mouse, *Vegfc* mutant LECs fail to exit the CV and cannot form mature vessels (Hägerling *et al*., 2013; Karkkainen *et al*., 2004). Similarly, in absence of Vegfc signalling LEC sprouting is blocked in zebrafish (Le Guen *et al*., 2014; Hogan *et al*., 2009a).

VEGFC also has a role in the regulation of LEC proliferation. In mouse, the overexpression of *VEGFC* causes lymphatic vessels hyperplasia (Jeltsch, 1997). In zebrafish, Vegfc regulates LEC progenitor numbers in the PCV (Koltowska *et al*., 2015a) by its effects on proliferation (Grimm *et al*., 2019; Helker *et al*., 2013).

At later stages, *vegfc* signalling is involved in the migration out of the HM. This function is mediated by the downstream effectors Sox18, Sox7 and Mafba (Koltowska *et al*., 2015b). These data together indicate that VEGFC signalling is in control of a number of key discrete cellular processes in LEC development, such as specification, migration and proliferation. However, the fact that the Vegfc-induced proliferation rate is unaffected in *mafba* mutants (Koltowska *et al*., 2015b) suggests that these processes depend on multiple, specific pathways, and not on a single downstream cascade.

PROX1 is the earliest marker of lymphatic identity

The transcription factor *Prospero-related homeobox 1* (*PROX1*) is the most prominent marker of LEC identity. In mouse, the gene is expressed by the LEC precursor population before sprouting out from the CV (Wigle and Oliver, 1999). Deletion of *Prox1* results in perinatal death and total absence of a lymphatic vasculature (Wigle and Oliver, 1999). During developmental stages, *Prox1* is also active in other tissues, such as the central nervous system, skeletal muscles, liver and retina, but within the endothelial cell population its expression is restricted to LECs.

Two paralogs of *Prox1*, called *prox1a* and *prox1b,* have been identified in zebrafish. *prox1b* is expressed in ECs and lymphatic progenitors from 24 hpf to 48 hpf. However, endothelial expression is lost at later stages, and both knock-down and knock-out *prox1b* embryo lymphatics develop normally, suggesting the gene is not necessary for lymphatic vascular development (Tao *et al*., 2011).

Zebrafish *prox1a* is expressed by the lymphatic progenitors before sprouting out from the PCV, and is therefore the earliest marker of lymphatic identity (Dunworth *et al*., 2014; Koltowska *et al*., 2015a). Live imaging has revealed that after the lymphatic progenitors in the dorsal PCV undergo a cell division, one of the daughter cells upregulates *prox1a* expression and starts the sprouting process, while the other downregulates it and stays in the PCV (Koltowska *et al*., 2015a) (**Figure 3**). Standard *prox1a* mutants show a marginal reduction in LECs (van Impel *et al*., 2014; Koltowska *et al*., 2015a), but in maternal zygotic mutants the number of lymphatic progenitors is drastically reduced, suggesting that maternal depositions can compensate the loss of *prox1a* (Koltowska *et al*., 2015a).

The upstream regulation of *PROX1* in LECs has been investigated in detail in mammals (Francois *et al*., 2011). In mouse, SRY-box transcription factor 18 (SOX18) and COUP transcription factor 2 (COUP-TFII) act as important *PROX1* regulators. SOX18 binds to the *PROX1* promoter to activate its transcription, and it is expressed by the cells in the CV that will later become LECs (François *et al*., 2008). COUP-TFII acts upstream of *Prox1*, binding to its regulatory regions (Srinivasan *et al*., 2010) and inducing its expression in LECs (Lin *et al*., 2010; Srinivasan *et al*., 2007). *In vitro*, COUP-TFII interacts with PROX1 to form heterodimers, which activate LEC transcriptional programmes (Aranguren *et al*., 2013) and regulate *Prox1* during LEC identity acquisition (Srinivasan *et al*., 2010).

In fish, however, the role of these factors in lymphatic development is contentious. While lymphatic defects and a reduction of *prox1a* expression can be observed in *coup-TFII* morphants (Aranguren *et al*., 2011), *coup-TFII* mutants are viable and their lymphatics develop normally (van Impel *et al*., 2014). Similarly, no vascular defects are found in *sox18* mutants (van Impel *et al*., 2014). Combinatory knock-down (Herpers *et al*., 2008; Pendeville *et al*., 2008) and knock-out (Hermkens *et al*., 2015) studies demonstrated that *sox7* plays a compensatory role in early arteriovenous specification, which is impaired if both *sox18* and *sox7* are lost. However, *sox18* and *sox7* are dispensable for Vegfc-induced *prox1a* expression, suggesting they are not involved in *prox1a* regulation in zebrafish (Koltowska *et al*., 2015a). Therefore, the actors involved in *prox1a* upstream regulation in zebrafish are yet to be discovered.

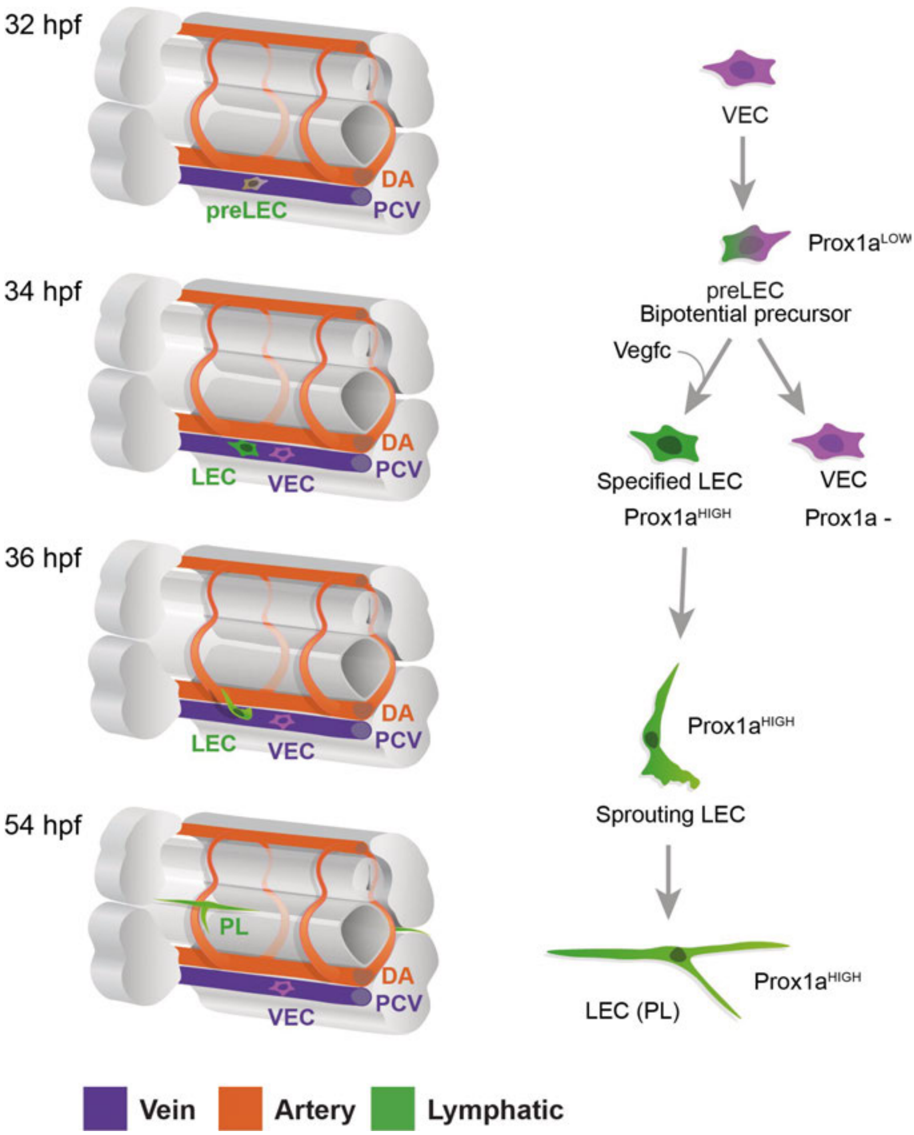

Figure 3: *prox1a* expression during early LEC specification. At 34 hpf, cells expressing low levels of *prox1a* can be seen in the PCV. These cells undergo a division, and one of the daughter cells upregulates *prox1a* expression, while the other remains a VEC. At 36 hpf, the *prox1a* positive LEC precursors sprout out of the PCV and migrate to the horizontal myoseptum, maintaining high *prox1a* expression levels. DA: dorsal aorta, LEC: lymphatic endothelial cell, PCV: posterior cardinal vein, PL: parachordal lymphangioblast, VEC: venous endothelial cell. Adapted from Koltowska *et al*., 2015a.

mafba regulates LEC migratory behaviours

During the early stages of lymphatic vascular development, the movement of LEC progenitors in the trunk can be divided in two separate phases: sprouting out from the PCV to the HM and migration from the HM to form the lymphatic vessels. A forward genetic screen identified the TF *v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog Ba* (*mafba)* as an important factor in regulating the correct migratory behaviour of LECs (Koltowska *et al*., 2015b). Prior to sprouting, *mafba* is expressed in the venous endothelium, and is later enriched in LECs. *mafba* embryos show a severe impairment of trunk lymphatic development, up to complete absence. However, the facial lymphatic network only shows a minor reduction, concentrated in the LAA and MFL. Mutant *Mafb* mice display a far less severe phenotype, resulting in viable adults with only a mild delay in lymphatic development and abnormalities of the diaphragm lymphatics (Dieterich *et al*., 2020; Rondon-Galeano *et al*., 2020).

MAFB is involved in the VEGFC signalling cascade in both mouse and zebrafish (Dieterich *et al*., 2015; Koltowska *et al*., 2015b), and in the latter it is situated downstream of SOXF (Koltowska *et al*., 2015b). However, in *mafba* mutants, the LEC progenitors correctly sprout out from the PCV and reach the HM, but fail to migrate out of the HM to form the lymphatic vasculature (**Figure 4**). *mafba* is therefore not involved in the Vegfc-dependent specification or sprouting, and only acts downstream of Vegfc in the cascade regulating migration, indicating that the different Vegfc-mediated processes might involve separate downstream effectors (Koltowska *et al*., 2015b).

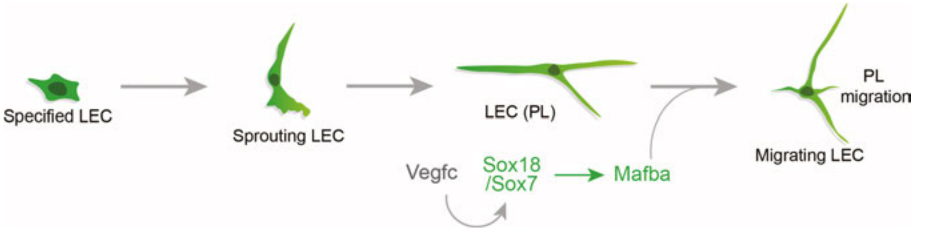

Figure 4: Role of *mafba* in trunk LEC migration. *mafba* plays no role in the early specification and spouting from the PCV, but is later needed for migration out of the HM. Adapted from Koltowska *et al*., 2015b.

GATA2, FOXC2, NFATC1 are regulators of lymphatic valve development

Lymphatic valves are a feature of mammalian lymphatics. They aid the lymph flow by preventing backflow and are located at regular intervals along the lymphatic vessels. Valve formation defects can cause lymphatic dysfunction in human pathological conditions (Petrova *et al*., 2004). Zebrafish lymphatic valves were only recently discovered (Shin *et al*., 2019). The only lymphatic valve described so far in this organism sits at the upper-branching point between the LFL and the FCLV. A lympho-venous valve has also been described in zebrafish at the anterior medial end of the FCLV, connecting the lymphatic network to the venous circulation (Shin *et al*., 2019).

Lymphatic valve development depends on TFs such as *PROX1*, *GATA binding protein 2* (*GATA2*), *forkhead box C2* (*FOXC2*) and *nuclear factor of activated T cells 1* (*NFATC1*). *GATA2* is a zinc-finger TF of the GATA family. Mutations of this gene are connected to human Emberger syndrome, which leads to abnormal valve formation and lymphoedema (Kazenwadel *et al*., 2012; Ostergaard *et al*., 2011). *Gata2* physically binds to *Prox1* regulatory elements and is expressed in mouse lymphatic valves (Kazenwadel *et al*., 2015). In humans, oscillatory shear stress induces expression of *GATA2*, which is located upstream of the activation of *PROX1*, *NFATC1* and *FOXC2* transcription (Kazenwadel *et al*., 2012, 2015). Early *Gata2* deletion in the vasculature hinders the CV sprouting in mouse, but it does not affect LEC specification and *Prox1* expression (Frye *et al*., 2018). Deletion at later stages leads to oedema and defects in the formation of lymphatic and lympho-venous valves, accompanied by reduced *Prox1* expression (Geng *et al*., 2016; Kazenwadel *et al*., 2015). These results indicate that *PROX1* is regulated by *GATA2* contextually to valve formation, and not during the earlier lymphatic vasculature development. In zebrafish, *gata2a* is a marker for the developing lymphatic valve, and a valve-specific *gata2a* enhancer, located in intron 4, has been identified (Shin *et al*., 2019). In addition, mutants for both the *gata2a* coding sequence and the identified enhancer exhibit compromised valve morphology (Shin *et al*., 2019), confirming the crucial role of this TF in lymphatic valve formation across vertebrates.

Mouse *Foxc2* is essential for the later maturation of the lymphatic vasculature, but it has a negligible role in the early stages of lymphangiogenesis (Norrmén *et al*., 2009; Petrova *et al*., 2004). *Foxc2* is also necessary for lymphatic valve development, as *Foxc2*$^{-/-}$ embryos do not form valves (Petrova *et al*., 2004). A role for *Foxc1* in the forming valves has also been suggested (Fatima *et al*., 2016). In zebrafish there is no *FOXC2* homolog; however, *foxc1a* is co-expressed in VECs and LECs with *gata2a*. Moreover, partially rescued *foxc1a* mutants fail to form valves (Shin *et al*., 2019), suggesting this gene plays a similar role to *FOXC2* in valve development.

During development, *Nfatc1* is expressed in mouse LECs, and enriched in lymphatic valves (Norrmén *et al*., 2009). VEGFC signalling and PROX1 can regulate *NFATC1* expression, and the gene interacts *in vitro* with FOXC2 to regulate transcription. When NFATC1 activity is inhibited by cyclosporin A, abnormal lymphatic patterning and a reduced number of lymphatic valves can be observed (Norrmén *et al*., 2009). In zebrafish, *nfatc1* is initially expressed in the facial lymphatics at 2 dpf, and it is later enriched in the developing valve (Shin *et al*., 2019). *nfatc1* mutants do not form a lymphatic valve (Shin *et al*., 2019).

As just illustrated, *GATA2*, *FOXC1/2*, *NFATC1* and *PROX1* are important factors in lymphatic valve development. However, there are some important differences in their interaction between mouse and zebrafish. Studies in mouse have shown that *GATA2* lies upstream of the other factors, and induces their expression (Kazenwadel *et al*., 2012, 2015). In zebrafish, however, mutants for *prox1a*, *nfatc1* and partially rescued *foxc1a* fail to form valves and show reduced expression of *gata2a* (Shin *et al*., 2019), suggesting that *gata2a* is located downstream of them. It remains to be elucidated if these observations indicate a difference in the regulation of valve formation between mouse and zebrafish, or if the regulatory mechanisms of valve formation involve some positive feedback loop between these TFs.

Epigenetic regulation in lymphatic vessel development

During development, the progenitor cells of different lineages specialise, migrate and differentiate in order to form functional tissues and organs. Since the cells of an organism all share the same genome, the resulting diversity fundamentally depends on differential gene expression. Epigenetic regulation is the collective term describing the processes by which the expression of genes is differentially regulated by means of chromatin rearrangements. The term describes many phenomena, from local changes in chromatin state to histone modification to alterations of chromatin architecture, all of which are key in controlling the fine spatio-temporal gene expression that underlies organ and tissue development.

In general, it must be noted that very little of the epigenetic regulation involved in LEC development has been investigated. However, a number of different tools and techniques have been successfully applied to the characterisation of chromatin organisation and *cis*-regulation in other populations of endothelial cells, offering a strong starting point for investigations into LEC epigenetics (Panara *et al*., 2022).

Cis-regulatory elements and enhancers

In eukaryotic cells, the genomic material in the nucleus is organised around protein tetramers called histones to form a macromolecule known as chromatin. Chromatin can be found in two different states: heterochromatin, tightly packed to prevent the access of DNA binding factors to the DNA, and euchromatin, relaxed to expose genomic sequences to the surrounding environment. Chromatin switches between these two conformations by acetylation and methylation of histones, which cause the DNA to loosen or pull in a compact cluster, or by DNA methylation, which negatively regulates gene expression by recruiting transcriptional repressors and preventing protein binding. Euchromatin is associated with active gene loci, since in this accessible DNA conformation the TFs and the RNA-polymerase can access their binding sites, the so called *cis*-regulatory sequences (CREs), and start transcription.

Enhancers constitute a subtype of CREs, and can be located downstream, upstream and in the intronic regions of a locus. Differently from promoters, they do not directly bind the transcriptional machinery, but interact with the transcription start site (TSS) by the formation of a DNA loop structure (Su *et al.*, 1990). The distance between an enhancer and the gene it regulates can be significant, and there have been reported cases of enhancers acting in *trans* (Bashkirova and Lomvardas, 2019; Tomikawa *et al.*, 2020). These long-range genomic interactions are made possible by the 3D folding of chromatin, which can bring distal elements in close topological proximity. Often, a single gene is regulated by separate enhancers in different tissues (Long *et al.*, 2016). This phenomenon is particularly important during development, allowing the independent spatio-temporal regulation of genes. For this reason, the study of enhancers has been a central theme of developmental biology.

Enhancers and sequence conservation

Enhancers are enriched in TF binding sites, and are therefore subject to negative selective pressure against mutations, while the surrounding non-coding DNA is free to drift neutrally. Therefore, many of the early described enhancers are located in regions of non-coding DNA marked by high evolutionary conservation (reviewed in Kikuta *et al.*, 2007) (**Figure 5**). Although there are cases of enhancers maintaining similar regulatory functions even when their sequences have diverged past recognition (Fisher *et al.*, 2006; Wong *et al.*, 2020), developmental enhancers often show high levels of conservation (Woolfe *et al.*, 2004). The analysis of non-coding DNA conservation can therefore be used to identify enhancer sequences. Compared with molecular-based approaches, this method has the technical advantage of requiring only *in silico* work. However, this approach presents two main limitations: it cannot identify non-evolutionary conserved elements, and it does not allow the generation of lineage-specific data. This last drawback can be overcome by combining the analysis of non-coding DNA with other techniques, such as the Assay for Transposase-Accessible Chromatin with high-throughput sequencing (ATAC-seq). Sequence conservation analysis has been successfully employed to identify enhancers of endothelial genes such as *flt1* (Bussmann *et al.*, 2010), *etsrp* (Veldman and Lin, 2012), *notch1b* (Chiang *et al.*, 2017) and *gata2a* (Dobrzycki *et al.*, 2020; Shin *et al.*, 2019). Crucially, this method allows the identification of homologous enhancers in human and model animals, allowing the *in vivo* characterisation of their activity and possible pathogenic mutations.

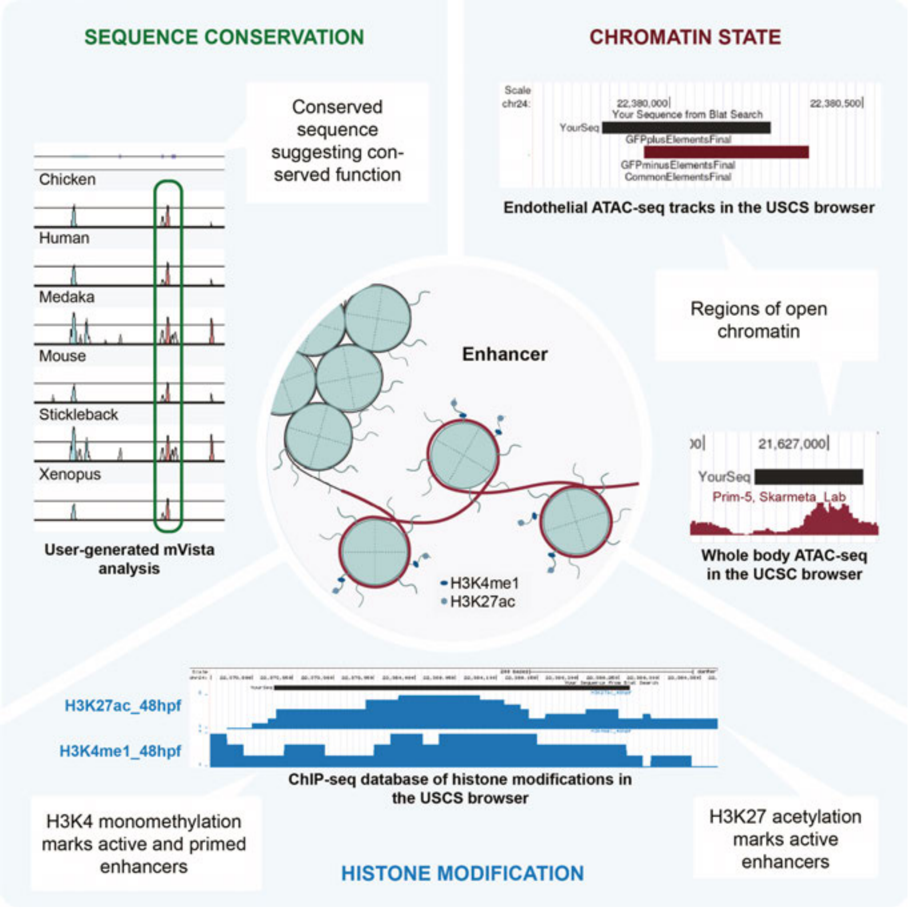

Figure 5: Schematic summary of the main markers of enhancer activity. Enhancer sequences can be detected on the bases of sequence conservation (green), chromatin state (red) and presence of histone modifications associated with CREs (blue). Adapted from Panara *et al*., 2022

Enhancers and chromatin accessibility

Active enhancers are located in accessible regions of chromatin, where they can be bound by TFs and other DNA-binding proteins in order to regulate gene expression. Therefore, the identification of regions of open chromatin through techniques such as ATAC-seq (Buenrostro *et al*., 2013) can be used for enhancer prediction (**Figure 5**). Briefly, this technique relies on a hyperactive Tn5 transposase for digesting and loading sequencing adapters preferentially in regions of open chromatin, where the DNA is exposed. Deep sequencing then allows the mapping of these fragments along the genome, identifying regions of relaxed chromatin. Due to the low input of cells required, ATAC-seq has become the go-to method for investigating chromatin accessibility, taking over previous techniques such as DNAse hyper-sensibility. One of the main advantages of using ATAC-seq for enhancer identification is the possibility of generating tissue specific datasets, revealing the presence of population-specific enhancers. In the last few years, a single cell approach (scATAC-seq) has also become available, allowing the characterisation of enhancers restricted to rare cell types.

Both bulk and scATAC-seq have been successfully used to characterise the chromatin state of endothelial cells (Panara *et al*., 2022). In zebrafish, a pan-endothelial bulk ATAC-seq database is available (Quillien *et al*., 2017), and it has been used to identify a number of endothelial enhancers for genes such as *mafba*, *mafbb*, *dll4*, *nrp1b* (Quillien *et al*., 2017) and *gata2a* (Dobrzycki *et al*., 2020). The latter enhancer was later also identified by an independent ATAC-seq experiment (Shin *et al*., 2019), showing the robustness of the method in identifying tissue-specific enhancers.

Enhancers and histone modifications

Different *cis*-regulatory elements, including enhancers, are marked by specific histone modifications. Histones are protein hetero-octamers around which the genomic DNA is wound, forming a structure called nucleosome. Histone modifications, such as methylation and acetylation of specific residuals, function as a readout of the chromatin state and correlates with the activity of the CRE element they mark (Chen *et al*., 2012; Fernández and Miranda-Saavedra, 2012). For example, active promoters are marked by a tri-methylation of the lysine 4 of histone 3 (H3K4me3) (Bernstein *et al*., 2002, 2005; Santos-Rosa *et al*., 2002; Wardle *et al*., 2006), while active enhancers are marked by both a mono-methylation of the lysine 4 of histone 3 (H3K4me1) and acetylation of lysine 27 of histone 3 (H3K27ac) (Bonn *et al*., 2012; Creyghton *et al*., 2010; Heintzman *et al*., 2007) (**Figure 5**). Moreover, enhancers that are primed for activation can be marked by H3K4me1 alone (Creyghton *et al*., 2010). Histone modifications can therefore provide important cues not only about the presence of a CRE, but also about its function and activation state.

A number of enzymes mediating histone modifications have been found to be involved in vascular development in zebrafish, confirming the importance of this epigenetic mechanism in endothelial cell development. The histone methyltransferase PR Domain containing 16 (Prdm16) is enriched in ECs and is necessary for their correct differentiation and migration (Matrone *et al*., 2021). The histone demethylases Kdm4a and Kdm4c have a role in vasculogenesis in both zebrafish and mouse by mediating the specification of EC progenitors (Wu *et al*., 2015). The histone acetyltransferase P300 is recruited to vascular loci by ERG (Kalna *et al*., 2019) and is involved in the regulation of key vascular genes such as *hlx1* and *dll4* (Fish *et al*., 2017). Protein Arginine Methyl Transferase 5 (Prmt5) has also been found to have a role in vascular formation, but independent from its methyltransferase activity. Instead, it promotes the looping of chromatin by acting as a scaffold protein, and mediates chromatin looping to regulate vascular factors such as *cdh5* and *fli1b* (Quillien *et al*., 2021). Together, these findings show the important role of histone modifications in the regulation of gene expression associated with vascular development.

Compared with sequence conservation and analysis of chromatin state, the use of histone modifications to identify endothelial enhancers has lagged behind. This is mainly due to a technical issue: for a long time, the main technique used to investigate histone modifications has been chromatin immunoprecipitation sequencing (ChIP-seq). However, this technique requires a very high input of cells, and is not feasible for a relatively rare population such as the zebrafish endothelium. Whole-body dataset for different zebrafish embryonic stages have been generated (Aday *et al*., 2011; Bogdanovic *et al*., 2012), but the dilution of the endothelial signal presents an issue for enhancer identification. Despite these limitations, the 48 hpf dataset (Bogdanovic *et al*., 2012) has been used to successfully characterise a *notch1b* arterial enhancer sequence (Chiang *et al*., 2017). Fortunately, the emergence in the last years of techniques such as CUT&RUN (Skene and Henikoff, 2017) and CUT&Tag (Kaya-Okur *et al*., 2019), which require far less input material, will allow the generation of endothelium-specific histone modification profiles in zebrafish.

Chromatin architecture and gene regulation

Within the nucleus, chromatin is organised hierarchically in a series of 3D interactions. At the lowest level, loops are formed to connect *cis*-regulatory elements to promoters (Liu *et al*., 2021) (**Figure 6**). Loop interactions are located within Topologically Associated Domains (TADs) (Dixon *et al*., 2012; Liu *et al*., 2021), regions of DNA that interact more internally than with the rest of the genome (**Figure 6**). TADs are sorted between two compartments, A and B, corresponding to macro-regions of active and inactive chromatin (Liu *et al*., 2021; Rao *et al*., 2014) (**Figure 6**). These multiple levels of tridimensional organisation have not only important structural and protective functions, but also play a fundamental role in regulating gene expression. Specifically, TADs have been shown to play a major role in *cis*-regulation, as most enhancer-promoter interactions happen within TADs since their borders work as insulators (Sexton and Cavalli, 2015). The physical structure of TADs is explained by the loop extrusion model (Hadjur *et al*., 2009; Rao *et al*., 2014; Splinter, 2006). At the boundaries of a TAD, clusters of CTCF-binding sites oriented in converging conformation are found. The CTCF proteins bound to these elements are able to recruit a cohesin ring. The DNA extrudes through the ring until it encounters the CTCF element at the opposite end of the TAD, forming an isolated domain. Within this domain, smaller loops can form to allow the interaction of *cis*-regulatory elements with their loci.

TAD organisation tends to be highly conserved, and the same topologically associated domains can be observed in different cell populations (Dixon *et al*., 2012; Rao *et al*., 2014) and across different vertebrate species (Harmston *et al*., 2017; Krefting *et al*., 2018). However, at a sub-TAD level the loop organisation can vary between tissues (Dixon *et al*., 2012; Rao *et al*., 2014), due to specific interactions between genes and their regulatory elements. Recent studies also casted doubt on the absolute stability of TAD formation. Single-cell approaches revealed that the conformation of TADs is more variable than previously thought (Bintu *et al*., 2018; Finn *et al*., 2019). Moreover, there is increasing evidence that TADs can behave plastically during developmental stages, changing their boundaries during cell fate acquisition (Boya *et al*., 2017; Ringel *et al*., 2022). These observations suggest that TAD plasticity might contribute to the complex gene regulation occurring during development.

In order to characterise the TAD and loop structure of a tissue, techniques such as Hi-C are commonly used (Belton *et al*., 2012). The genomic material is first crosslinked with formaldehyde to fix the DNA interactions, and then shredded. The fragmented DNA is then biotinylated, ligated and sequenced. Because of the crosslinking, DNA regions interacting in 3D will remain bound together and ligated to form hybrid sequences that align to both interacting regions. By mapping these “hybrids”, it is possible to reconstruct the presence of long-range genomic interactions, such as loops and TADs. Hi-C and other closely-related techniques have been successfully used in zebrafish (Franke *et al*., 2021; Kaaij *et al*., 2018; Wike *et al*., 2021; Yang *et al*., 2020) as well as in human endothelial cell samples (Higashijima *et al*., 2020; Ma *et al*., 2022; Nakato *et al*., 2019; Papantonis *et al*., 2012), were they led to the identification of a distal endothelial *KLF4* enhancer (Maejima *et al*., 2014). However, to this day Hi-C has not yet been used to characterised the chromatin organisation of the zebrafish endothelium.

The development of techniques such as Hi-C has opened the door for the investigation of chromatin architecture and long-range interactions, and their connection with local *cis*-regulatory mechanisms. The integration of chromatin organisation, the activity of *cis*-regulatory elements, the transcription factors binding to them and the upstream signalling inducing these changes is one of the upcoming challenges for the field and key to fully understand differential gene expression during development.

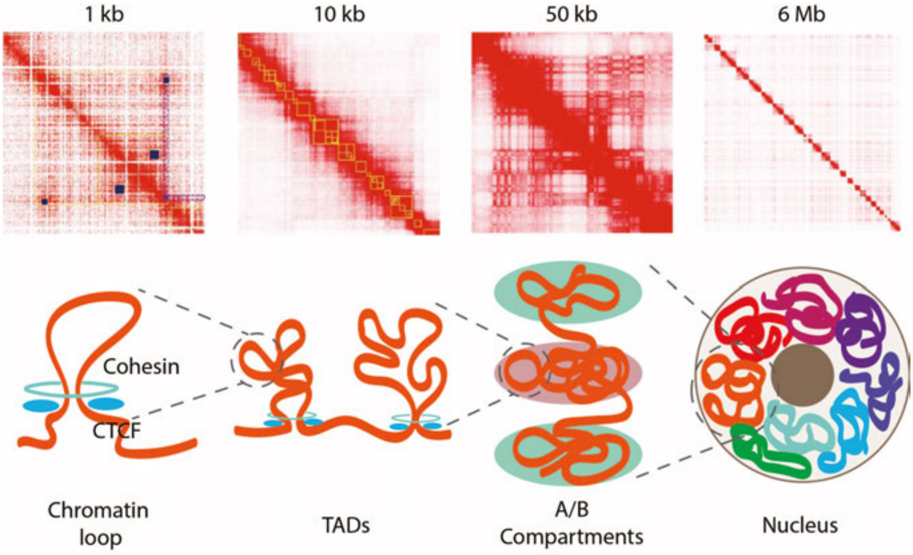

Figure 6: Hierarchical levels of chromatin organisation. At the highest resolution level, chromatin loops connect promoters with their enhancers in a CTCF-mediated mechanism. TADs collect and isolate chromatin loops insulating them from the surrounding regulatory landscape, and are also usually formed in a CTCF-dependent way. TADs are divided in A and B compartments, corresponding to regions of active and inactive chromatin in chromosomes. Adapted from Liu *et al*., 2021.

Present investigations

Paper I

Despite the wide morphological variety across the lymphatic network, little is known about the molecular differences in the developmental processes among these LEC populations. Zebrafish Mafba is necessary for lymphatic network formation in the trunk. *mafba* mutant LECs correctly exit the PCV but are unable to migrate out of the HM, leading to the total absence of trunk lymphatic vessels. However, the facial lymphatics develop with only minor defects in these embryos, suggesting differential requirements for *mafba* in the different lymphatic beds. In this study, we investigated the role of the *mafba* paralog *mafbb* in lymphatic development. Single *mafbb* mutants show no lymphatic phenotype. However, when both *mafba* and *mafbb* are knocked-out, the embryos develop a severe facial lymphatic phenotype, suggesting that *mafbb* can compensate for the loss of *mafba* in the head, but not in the trunk.

Knowing that *mafba* acts in a later phase of LEC development, we tested if LEC progenitor specification was also unaffected in the lymphatics of double mutants. Indeed, we found that the number of specified progenitors, marked by *prox1* expression, does not vary between mutants and siblings, suggesting *mafbb* also acts during the subsequent migration phase. The double mutant phenotype was further characterised, finding a reduction in the volume of the facial lymphatic vessels, as well as defects in lymphatic valve formation, which lead to leakage into the FCLV. Moreover, the ability of the lymphatic endothelium to drain the interstitial fluid is compromised in double mutants, suggesting their facial lymphatics cannot regulate fluid homeostasis. *mafba* has been shown to be regulated in the lymphatics by the Vegfc-SoxF axis in the trunk lymphatics. We therefore investigated if either *mafba* or *mafbb* were under the control of the same factors in the facial lymphatics. Using a quantitative real-time PCR approach, we examined the expression level of *mafba* and *mafbb* in sorted facial LECs of morphant embryos. We found that both *mafba* and *mafbb* expression is reduced in *vegfc* morphants. Moreover, *mafbb*, but not *mafba*, is regulated by SoxF in the facial lymphatics, suggesting an unexpected difference in regulation between the facial and trunk lymphatics. As *mafba* mutants fail to exit the HM, we characterised the migration of the facial lymphatic sprout. By live imaging the sprouting process, we could track the trajectories of leading and follower LECs. We found that the cell speed was unaffected in double mutants. However, the overall distance covered was reduced, and the directionality of migration was altered. These data suggest that the defects in facial lymphatic migration in double mutant embryos are not due to a compromised cell mobility, but to the inability to correctly sense the environment and orient migration.

In summary, we have shown that the lymphatic vasculature in face and trunk differs in its requirements depending on its topographical location, suggesting the presence of distinct transcriptional profiles.

Paper II

Prox1 is a transcription factor playing a central role in the development of several tissues and organs, including liver, pancreas, retina, heart, central nervous system and lymphatic endothelium.

In this study, we identified a *Prox1* enhancer, *-11Prox1*, driving expression in the lymphatic endothelium, and later specifically in the lymphatic valve of mice. The enhancer is evolutionarily conserved in zebrafish, where it drives expression in the lymphatic valve. The mouse element is bound by GATA2, FOXC2 and NFATC1, and binding sites for the last two factors are present in the homologous zebrafish sequence, called *-2.1prox1a*. Deletion of different fractions of the enhancer in mouse caused reduced litter size and often perinatal death, accompanied by lymphatic abnormalities and interstitial oedema. When the earlier phenotypes were investigated, oedema was found at E14.5, while blood-filled lymphatics were observed around E18. A lack of valves in the skin and mesenteric lymphatics was also observed. The LECs of mutant embryos moreover showed reduced levels of Prox1, and a transcriptional profile skewed towards blood endothelial cells (BECs). The observed phenotypes were stable as long as the GATA2 binding site in the enhancer was knocked out. The ability of the other predicted transcription factors, such as NFATC1, FOXC2 and PROX1, to bind the enhancer in the absence of the GATA2 site was therefore investigated. It was found that none of these proteins can physically bind *-11Prox1* in the absence of GATA2, which therefore covers a key role in the activity of the enhancer. An additional phenotype observed in the enhancer mutants was the presence of RUNX1 positive cell clusters in the jugular sacs, which also exhibited other markers of haematopoietic stem and progenitor cells. When plated to assess haematopoietic stem and progenitor cell activity, mutant RUNX1 positive cells generated colonies enriched in hematopoietic markers and depleted of LEC markers. Interestingly, the RUNX1 positive cell clusters could also be observed in wild-type embryos, although they were rarer, and generated smaller colonies when plated. Together, these data suggest that LECs can transition to assume a hematopoietic identity through a *PROX1*-dependent mechanism.

In summary, we have discovered an evolutionary conserved *PROX1* enhancer necessary for correct lymphangiogenesis in mouse. Moreover, this work discovered a previously unknown role for *PROX1* in repressing the hematopoietic identity in LECs.

Paper III

The transcription factor *Prox1* is an early marker of lymphatic identity, and it is necessary for lymphatic development across vertebrates. *Prox1* knock-out causes the de-differentiation of LECs into BECs in mouse, suggesting a key role for this gene in establishing LEC identity. However, no detailed characterisation of *Prox1* control over the transcriptional profile of LECs has been conducted so far. Zebrafish have two *Prox1* paralogs, called *prox1a* and *prox1b*, which are necessary for the correct development of the lymphatic vascular system.

In this study, we investigated how both the transcriptome and the chromatin accessibility of LECs changes at different points during development in the absence of *prox1*. First, a wild-type atlas of endothelial cells was generated by FAC sorting and scRNA-seq, and the LEC clusters were identified and characterised. *prox1a* has been shown to be necessary for the correct formation of lymphatic vasculature. We collected the endothelial cells of *prox1a* mutant embryos and sequenced their transcriptomes. A comparison with the WT dataset revealed that mutant LECs fail to completely differentiate, and are found in an intermediate cluster between WT LECs and VECs. This is in line with previous observations in mouse, confirming the role of *Prox1* in maintaining lymphatic identity. In order to characterise the chromatin changes accompanying this transcriptomic shift, a scATAC-seq database was generated, using both WT and *prox1a* mutant cells at 4 dpf. Interestingly, no *prox1a* mutant EC could be localised in the LEC cluster on the base of chromatin alone, which suggests deep epigenetic changes accompany the loss of *prox1a* in LECs. Motif enrichment was run on the differentially accessible regions in WT LECs and identified binding sites for known LEC regulators. Surprisingly, in the *prox1a* mutant ECs we found a number of regions more accessible than in both the VEC and LEC clusters. These regions were enriched for binding sites for factors involved in early vasculogenesis and haematopoiesis, suggesting that the depletion of *prox1a* can reactivate gene networks involved in alternative cell fates.

In the previous experiments we considered zygotic *prox1a* mutants, in which compensation from *prox1b* as well as maternal contributions of *prox1a* still allow partial LEC formation and migration to the HM. In order to better characterise the effects of a complete loss of *prox1* on the early specification of LECs, we generated a scRNA-seq dataset of 40 hpf endothelial cells on maternal zygotic (MZ) double mutants for *prox1a* and *prox1b*. This time point allowed us to capture the moment LECs have just been specified and are starting to leave the PCV. We observed a general upregulation of genes connected to the VEC and hematopoietic fates in *MZprox1a/MZprox1b* mutants, but little indication of genes being positively regulated by *prox1*.

Overall, our study data suggests that the role of *prox1* in LEC development is mainly a repressive one, inhibiting alternative cell fates, rather than actively promoting LEC-specific factors.

Paper IV

The TF *PROX1* is a fundamental gene for the development of lymphatic vasculature. However, *PROX1* has a wide expression pattern during development, including tissues such as liver, pancreas and the central nervous system, suggesting *cis*-regulation may play a role in controlling the expression in a tissue-specific manner.

In this paper, we investigated the *cis*-regulatory landscape surrounding *prox1a*, using both a sequence conservation and a chromatin accessibility approach. We identified a number of enhancers driving different patterns of expression in the lymphatic vasculature. Due to the conserved role of *PROX1* in vertebrates, we hypothesised that the enhancers involved in lymphatic development might be evolutionarily conserved. We scanned the non-coding regions surrounding the locus and tested a number of conserved putative enhancers *in vivo*. Two enhancers driving reporter expression in subsets of the lymphatic vasculature were identified: -2.*1prox1a*, expressed in the facial lymphatics and especially in the lymphatic valve, and +15.*2prox1a*, expressed in the facial lymphatics but more predominantly in the FCLV. -2.*1prox1a* was further dissected to identify the core enhancer sequence, containing binding sites for FOXC2, NFATC1 and GATA2. None of the identified conserved enhancers was able to drive reporter expression in the trunk lymphatic vasculature. We therefore used a scATAC-seq approach to identify regions of open chromatin surrounding the *prox1a* locus. We identified a number of LEC-specific open chromatin sequences upstream of *prox1a* able to drive expression in the facial and trunk lymphatics. We then decided to test the biological necessity of one of the identified enhancers for the correct development of the lymphatic vasculature. We used CRISPR/Cas9 to delete the -*2.1prox1a* enhancer, whose expression is concentrated in the lymphatic valve. In mutants, the morphology of the valve was compromised, resulting in a leaky valve, indicating that the -*2.1prox1a* enhancer plays a role in regulating the correct development of this structure.

In conclusion, we characterised a number of *prox1a* enhancers, both conserved among vertebrates and not, driving expression in the lymphatic vasculature. Interestingly, some of the sequences can drive reporter activity in specific lymphatic subsets, such as the valve or the FCLV, suggesting a requirement for an independent, fine regulation of *prox1a* in different parts of the lymphatic vasculature.

Paper V

Differential gene expression is the basis of tissue and organ development. Changes in chromatin organisation dictate accessibility to gene regulatory elements and their interaction with promoters, and are therefore a key feature for organ formation. During lymphatic development, LECs differentiate from VECs and modify their expression profiles upregulating known lymphatic markers. However, the changes in chromatin structure that accompany this transition have not been characterised so far.

In this study, we applied a series of techniques such as Hi-C, ATAC-seq and scRNA-seq to characterise the chromatin and transcriptional profile of LECs. We identified over 1000 chromatin loops that are specific for LECs compared to BECs, including loops located close to the loci of known vascular regulators such as *kdr* or *prox1a*. Over 10.000 peaks of differentially accessible (DA) chromatin in LECs were identified as well. Combining these data, we could classify the kind of elements that were interacting through these loops, finding that loops in LECs were enriched for promoter-intron interactions and depleted for promoter-distal elements interactions when compared to BECs. Overall, these results show the presence of a specific chromatin organisation signature in LECs, which can provide hints for the further characterisation of novel lymphatic regulators. In order to identify candidate genes of interest, we performed scRNA-seq on VECs and LECs at 5 and 7 dpf. This led to the identification of a panel of genes significantly enriched in LECs at these time points. ATAC-seq data was used to identify putative enhancer sequences laying in the immediate vicinity of these candidates. The analysis returned a previously described *cdh6* enhancer, as well as four novel enhancers driving expression in the lymphatic endothelium *in vivo*. This confirms the viability of the generated dataset for the characterisation of LEC-specific *cis*-regulatory elements. We then asked if the LEC chromatin loops we characterised could be used to identify long-range enhancers acting in LECs. We filtered our data for loops marked by an open promoter at one end and a DA peak of open chromatin at the other end, and looked for genes enriched in LECs compared to BECs. We could identify a number of interactions which we assigned to be putative long-range enhancers, including some involving known lymphatic regulators, such as *mafba* and *tbx1.*

This work investigated the differences in chromatin organisation between LECs and BECs. The two populations are marked by a different distribution of chromatin accessibility, which exposes several different genes and regulatory elements to the binding of TFs and other DNA binding proteins, activating or inhibiting the expression of specific genes. At the same time, tissue specific chromatin loops bring different genomic regions in the 3D architecture of the nucleus in close contact, allowing tissue-specific interactions between loci and their regulatory sequences. We were able to map tissue-specific loops and associated differentially accessible chromatin regions to the *loci* of known lymphatic regulators, such as *tbx1* and *mafba*, confirming that these processes of chromatin remodelling, and associated changes in gene expression, accompany the segregation of LECs and VECs.

Future perspectives

Organo-typicity of the lymphatic vasculature

The topic of organo-typicity, which is to say the heterogeneity of a tissue depending on its specific microenvironment, has been rising to prominence in the vascular field in the last few years. During my PhD studies, I found evidence of an early developmental origin for such differences within the zebrafish lymphatic network. The differential ability of *mafbb* to compensate for *mafba* loss between head and trunk in **Paper I** suggests a fundamental difference between these two LEC populations. Similarly, the identification of a *prox1a* enhancer expressed prevalently in the FCLV in **Paper IV** suggests this gene is being regulated differently in separate subunits of the network. Finally, in **Paper V**, we were able to identify genes, such as *cldn11a*, with a clear enrichment in facial LECs compared to the trunk, as well as enhancers, such as *+13tbx1* and -1*.2cxcl12a*, with a spatially restricted expression pattern. Together, these findings hint at the early establishment of deep transcriptomic and possibly epigenetic differences between distinct LEC populations, depending on their anatomical location. Such spatially restricted enhancers represent excellent candidates for the generation of genetic tools targeting specific lymphatic vascular beds. Moreover, the study of these topologically delimited elements could shed light on important differences in the molecular signature of distinct lymphatic vasculature beds and provide possible candidates for targeted therapeutic approaches.

Multi-enhancer regulation of *prox1a* in LECs

PROX1 is one of the earlier markers of LEC identity across vertebrates and plays a crucial role in lymphatic development in both mouse and zebrafish. As this gene is expressed in many other tissues during development, we investigated the non-coding regions surrounding it in **Paper II** and **Paper IV**, looking for lymphatic enhancers. *prox1a* is located within a large gene desert rich in conserved non-coding DNA elements, suggesting a complex *cis*-regulatory landscape. In our study, we could identify both conserved and non-conserved regulatory elements driving expression in LECs, suggesting that even within the boundaries of lymphatic development, the regulation of *prox1a* requires the concerted action of multiple enhancers to orchestrate the formation of a functional network. In our study, we characterised the phenotype resulting from the deletion of the *-2.1prox1a* enhancer, showing its importance for correct valve development. However, further studies would be needed to establish the relative role of each identified enhancer in the development of lymphatic vasculature, and the possible interactions between them.

A repressive function for *PROX1*

Paper II and **Paper III** both contributed important findings to the repressive function of *Prox1* in the establishment of LEC identity. In **Paper II**, we showed how the reduction in *Prox1* expression leads to the transition of some LECs in the jugular sac to a hematopoietic-like identity.

In **Paper III**, we demonstrated that a reduction in the level of *prox1a* is accompanied by an increase in accessibility to regulatory elements enriched for TF binding sites for known vasculogenesis and hematopoietic markers. Moreover, in single *prox1a* and double *prox1a/prox1b* mutants a reduction of LEC markers and an enrichment for venous markers could be observed.

During development, part of the endothelial population and the hematopoietic population derive from a common progenitor, the hemangioblast. At later stages, the LEC progenitors are specified from VECs, acquiring their specific identity. Therefore, the data in these two papers suggest that during lymphangiogenesis *Prox1* is involved in the repression of alternative cell fate, and that in its absence the cells somehow revert, or transdifferentiate, to other developmentally-close types. This repressive action of *Prox1* in LECs remains to be further investigated on a mechanistic level, in order to characterise the other molecular agents involved in this process.

Cis-regulation and chromatin organisation in LECs

With the rise in popularity and accessibility of techniques such as ATAC-seq and Hi-C, it has become possible to investigate the chromatin changes associated with developmental processes. In **Paper V**, we generated whole-genome datasets characterising the chromatin state and chromatin interactions of LECs and BECs, as well as the single-cell transcriptomic profile of LECs and VECs. These datasets represent a great tool to be used by the wider lymphatic community. As we show in our paper, the datasets allowed the identification of novel LEC identity markers, both general and topologically restricted. Moreover, the datasets were used to characterise undescribed enhancers, both on the basis of chromatin state alone, as well as chromatin interactions. Enhancers have in the last few years become an object of interest in the study of genetically transmitted diseases. Mutations in enhancers can have dramatic effects on the expression of key genes for cellular identity and homeostasis (see **Paper II**). However, many enhancers remain undescribed or uncharacterised, making the association between mutations and pathology extremely hard. In a future prospective, these data could be used to easily verify *in vivo* the activity and functional relevance of enhancers associated with lymphatic pathologies such as lymphoedema in humans, where these elements would be conserved either by sequence or by synteny.

Conclusions

In conclusion, this thesis has attempted to describe the different molecular processes involved in LEC development. However, understanding the global picture these data paint is not an easy task. One could compare the development of a cell to the story of *The blind sages and the elephant*. When stumbling upon an elephant on their way, the blind sages try to understand what stands in front of them, but as each one is touching a different part of the animal, they cannot agree on what is blocking the road. None of them is wrong, but they fail to realise they are each describing a single aspect of a complex entity that can only be correctly understood within the context of all the other cues. Likewise, the aspects of LEC development investigated in this thesis, from differential gene expression, to DNA accessibility, to chromatin architecture, all represent different points of view that describe the same "elephant": the acquisition of lymphatic endothelial cell identity. In order to avoid the sages' mistake, I tried to consider the LEC as the complex system it is. Thus, this work represents a first step in integrating the different perspectives on lymphatic vessel development to fully understand the morphological and molecular mechanisms driving it.

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