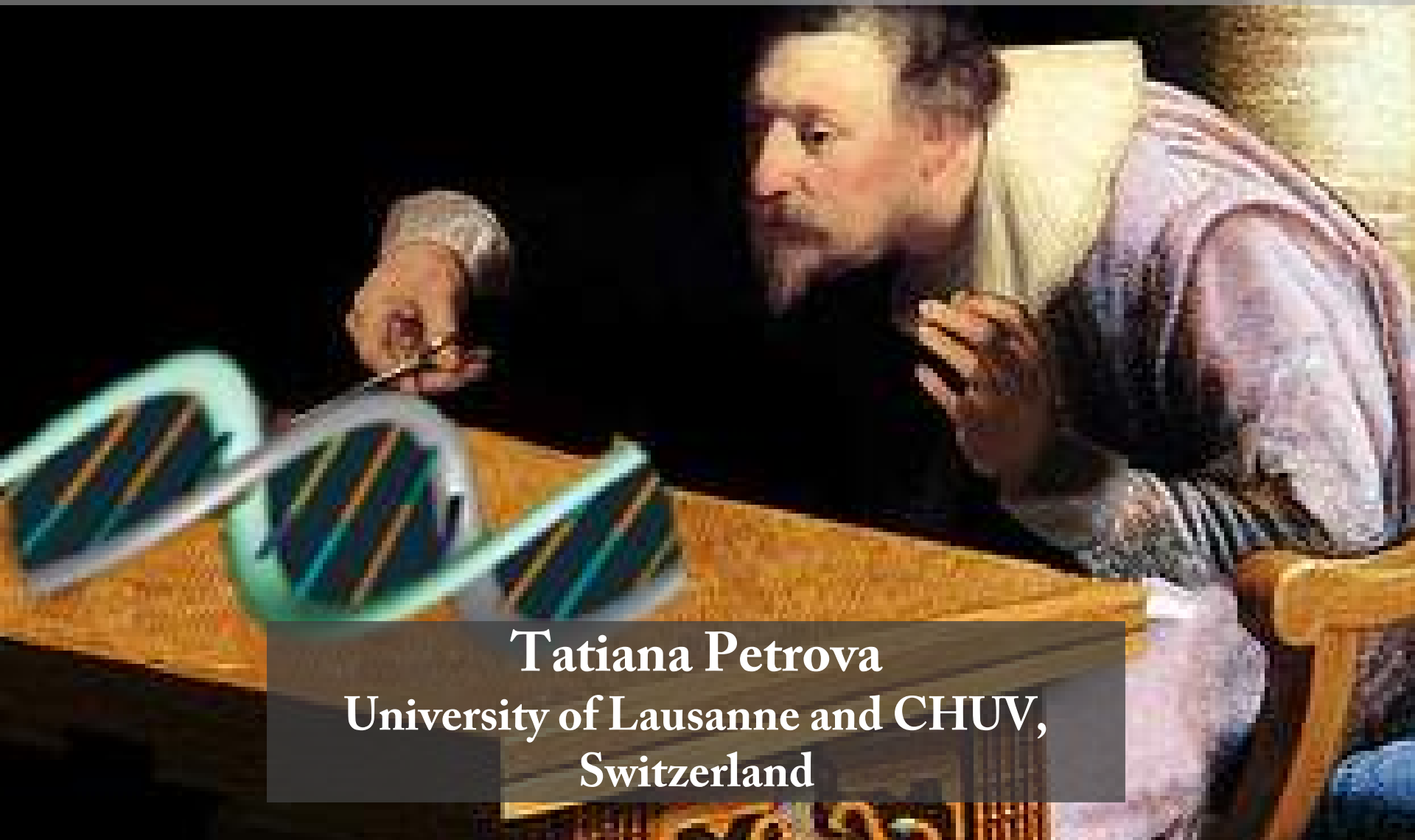
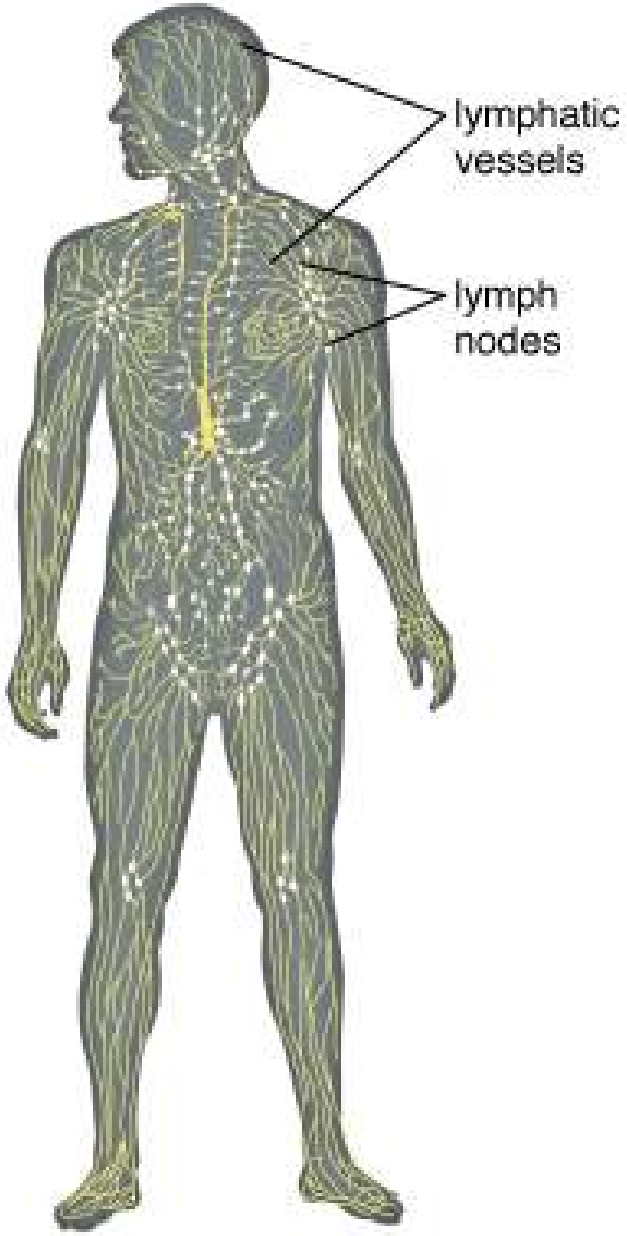
Dissecting the biology of lymphedema-distichiasis

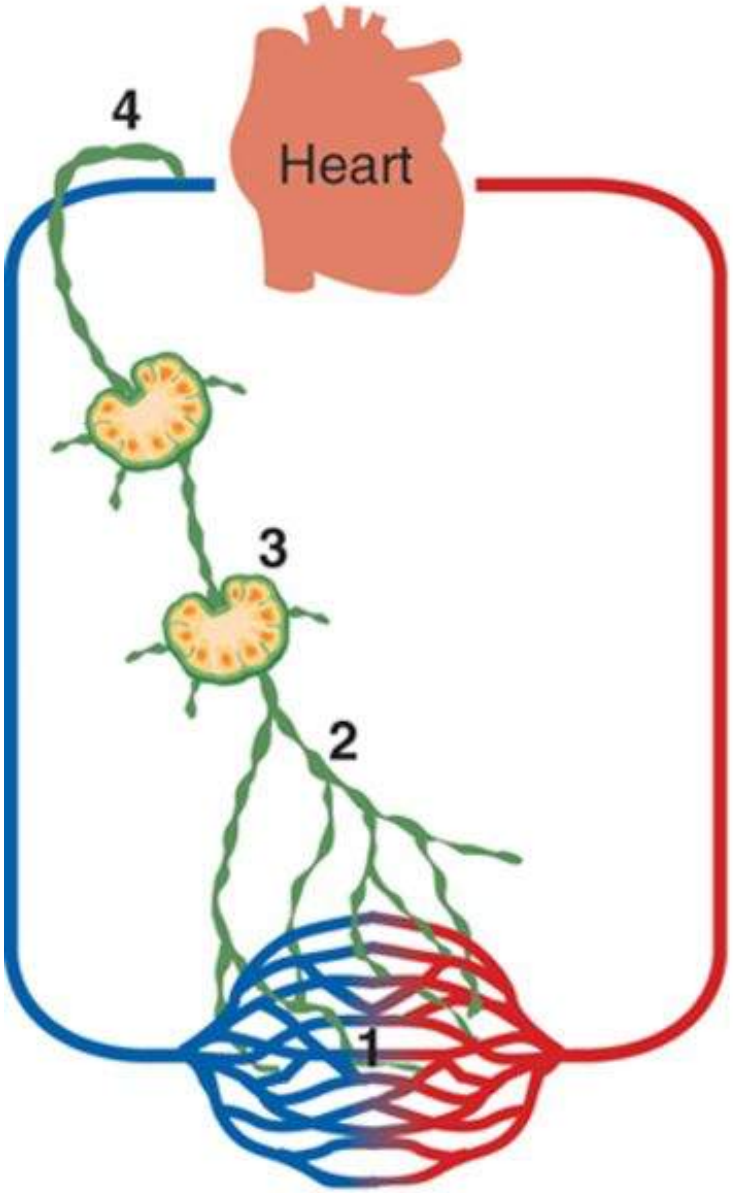


Tatiana Petrova
University of Lausanne and CHUV, Switzerland

The lymphatic system

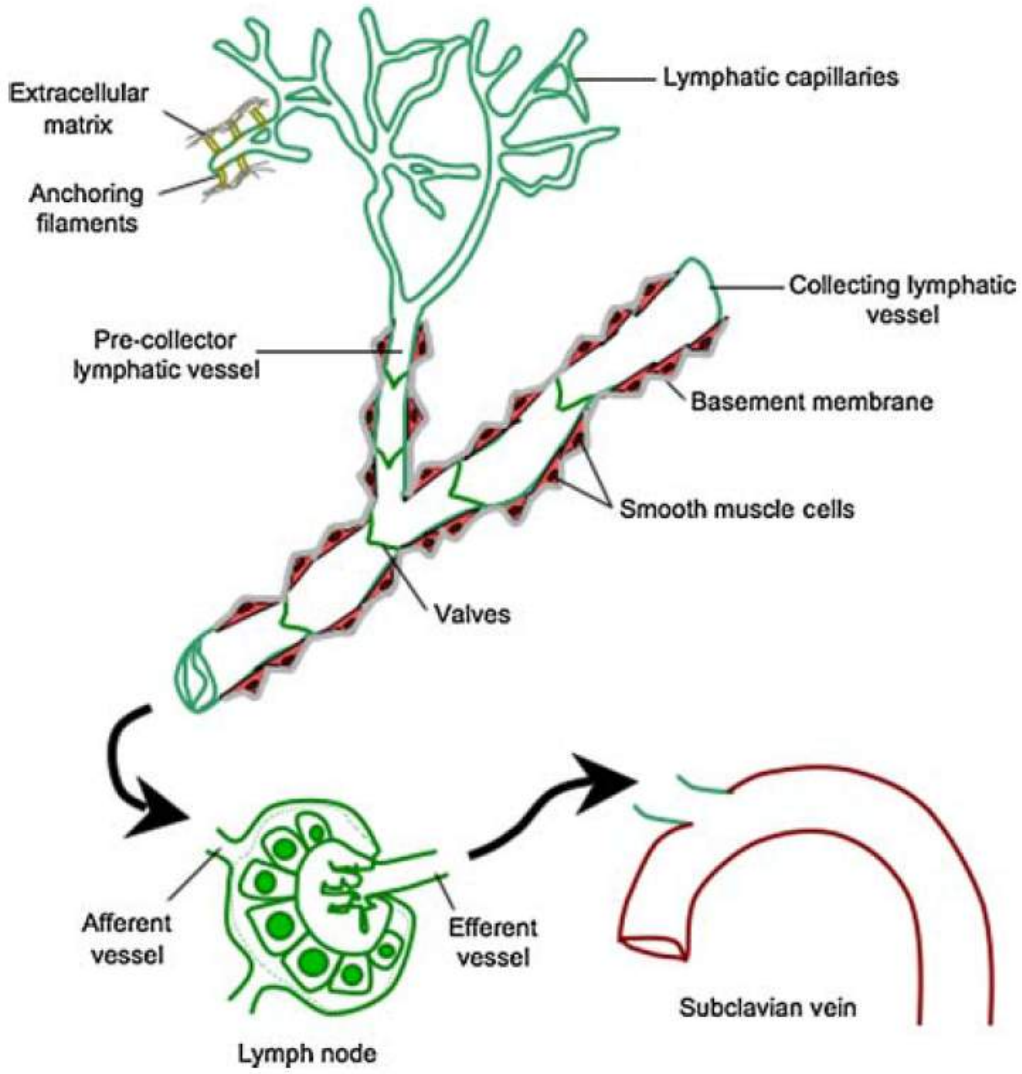


(c) Encyclopaedia Britannica, Inc.



Aspelund et al, 2016, Circ Res

Functions of lymphatic vasculature



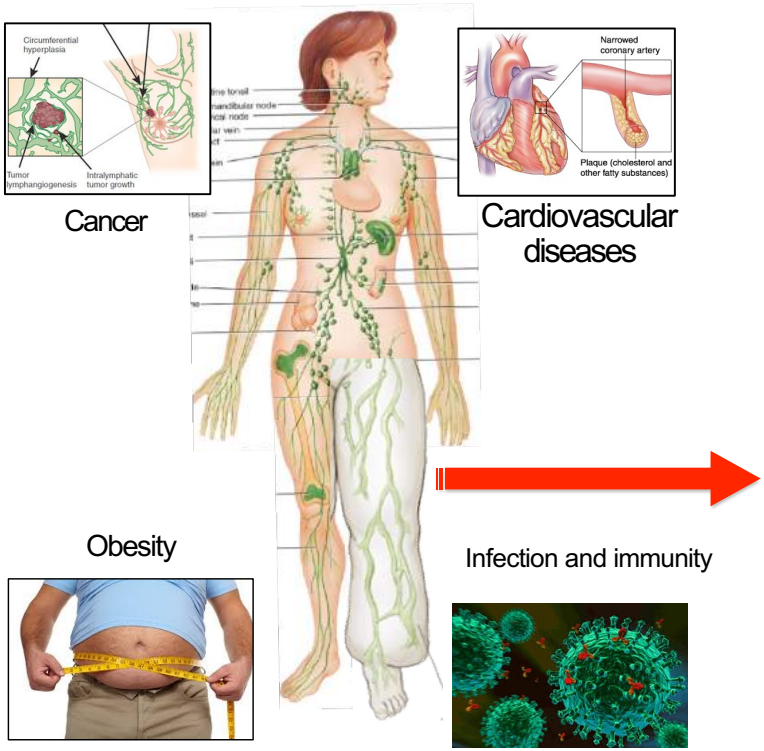
Interstitial fluid balance
Returning excess interstitial fluid to the blood circulation

Lipid transport
Absorption of fat and fat-soluble vitamins from the digestive tract

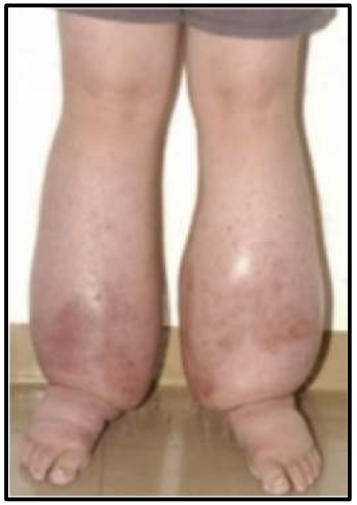
Immune surveillance
Carrying antigen and antigen-presenting cells

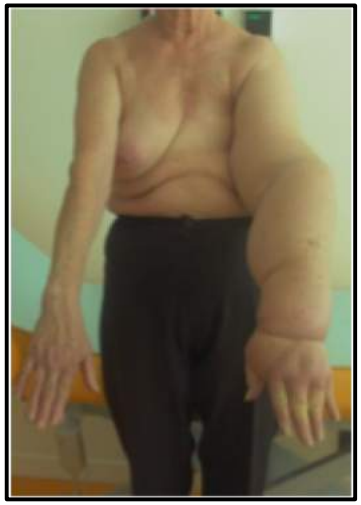
Maby-El Hajjami and Petrova, 2008, Histochem Cell Biol

Lymphatic vasculature and human diseases



Primary and secondary lymphoedema



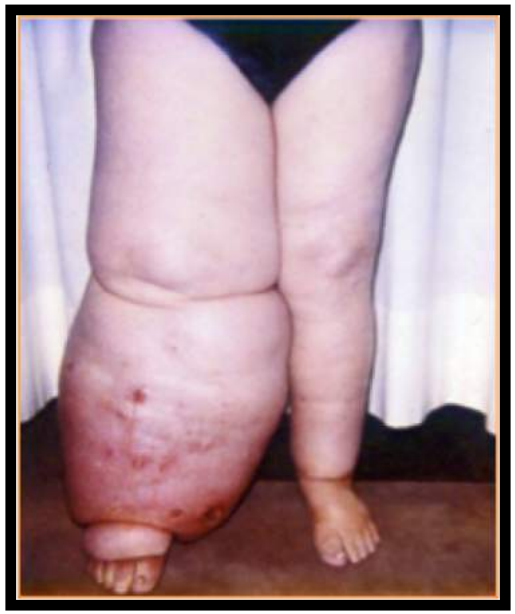


- 140 million cases worldwide
- Highly underestimated cause of disability comparable to Crohn's disease

Rare disease of lymphatic vessels

PRIMARY LYMPHEDEMA

- Chronic excessive tissue swelling
- Rare disease 1:6'000
- Autosomal dominant
- Affects up to 300'000 patients in Europe
- High morbidity



- Prone to infections
- Fibrosis
- Fat accumulation
- Sometimes lethal (lymphangiosarcoma, pulmonary edema or protein-loosing enteropathy)

Rare disease of lymphatic vessels

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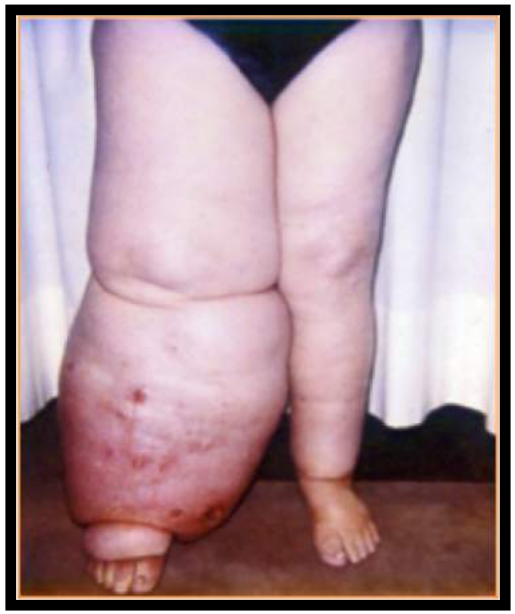
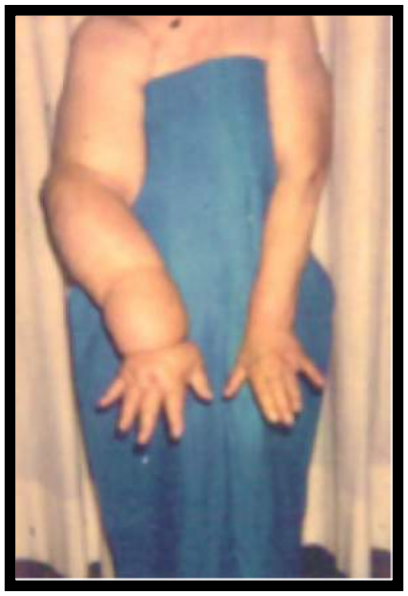


- Prone to infections
- Fibrosis
- Fat accumulation
- Sometimes lethal (lymphangiosarcoma, pulmonary edema or protein-loosing enteropathy)

SECONDARY LYMPHEDEMA

- Lymph node resection is the main cause
- Up to 30% of breast-cancer patients (350'000 individuals in Europe)
- Other cancers…



- May take several years to develop

↓

Genetic component?

Rare disease of lymphatic vessels
PRIMARY LYMPHEDEMA

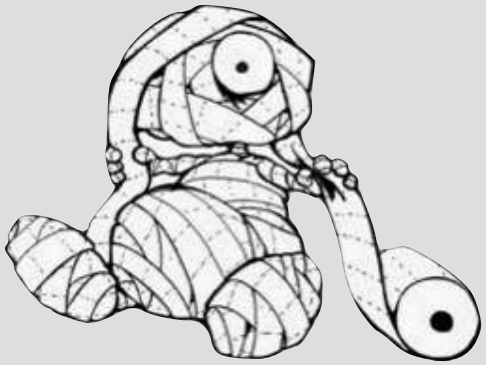
- Rare … ons
- Auto
- Affec … on
  patie
- Chro … al
  tissu … rcoma, … ma or
- High

S
L

- Lymp … years
  the m
- Up to … t?
  cance
  individuals in Europe)
- Other cancers…

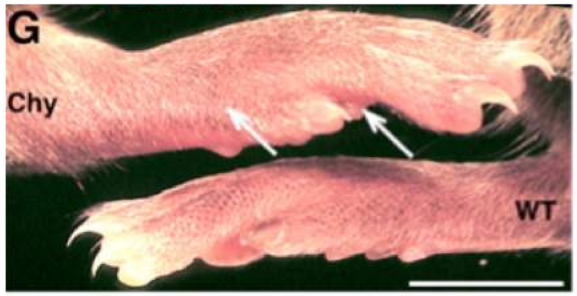
No curative treatment

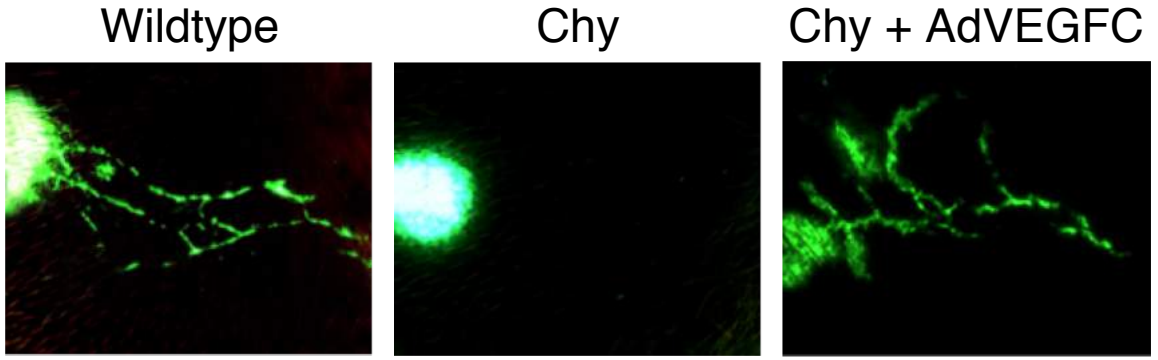
at the moment…
Only compression wraps
and regular manual lymphatic drainage!



Different subtypes of hereditary lymphedema

- 20 causative genes are identified so far
- **Example: Milroy's disease**
  -> mutations in *VEGFC* and *VEGFR3*
  -> congenital lymphedema
  -> *Chy* mouse model with mutations in Vegfr3
  -> hypoplastic cutaneous lymphatic vessels
  -> Adeno-VEGFC therapy re-grows lymphatic vessels and restores lymphatic flow



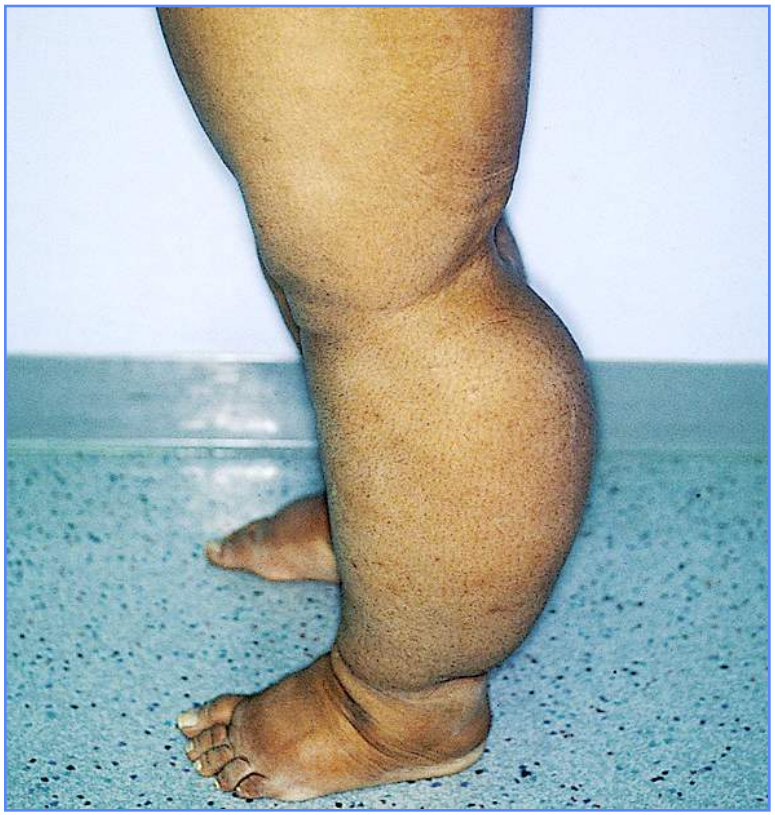


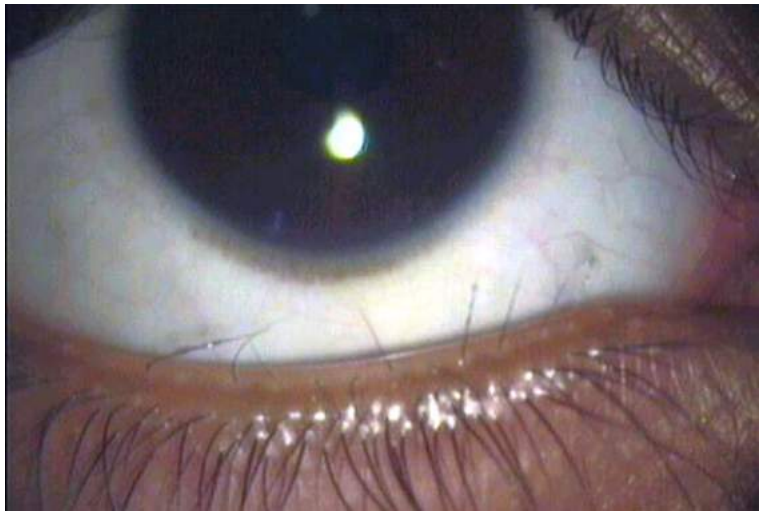
Karkkainen et al, 2001, PNAS

-> Lymfactin (AdVEGFC) is in clinical trial to treat secondary lymphedema (Herantis Pharma)

Lymphedema-distichiasis

- Autosomal dominant disease with reduced penetrance
- Late onset lymphedema (av.13 years old)
- Distichiasis - double row of eyelashes

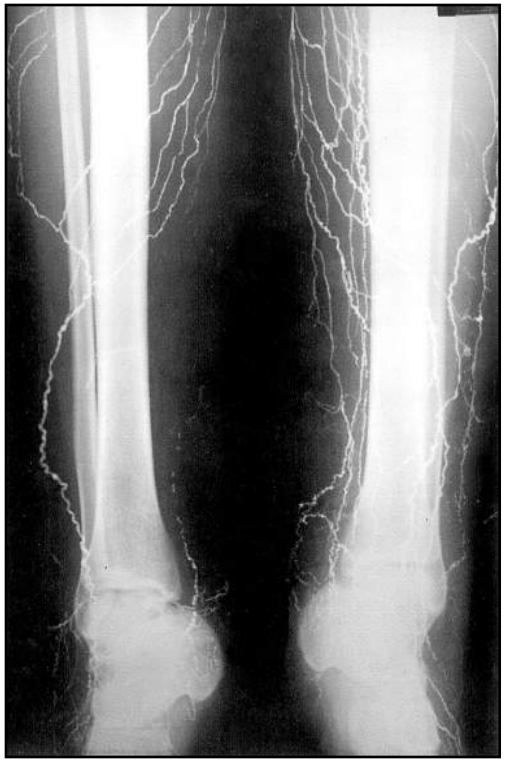


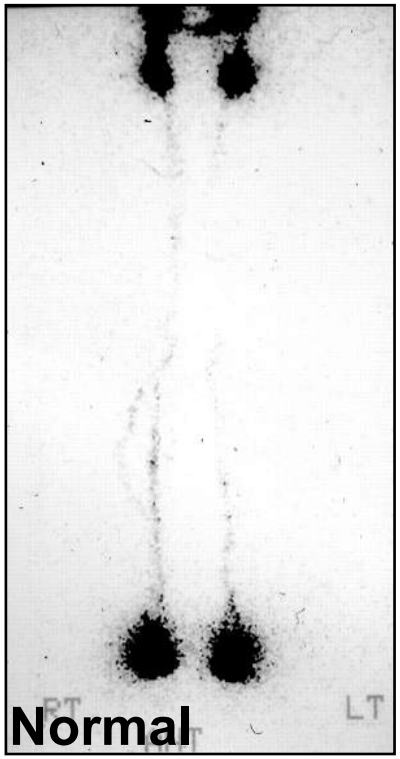


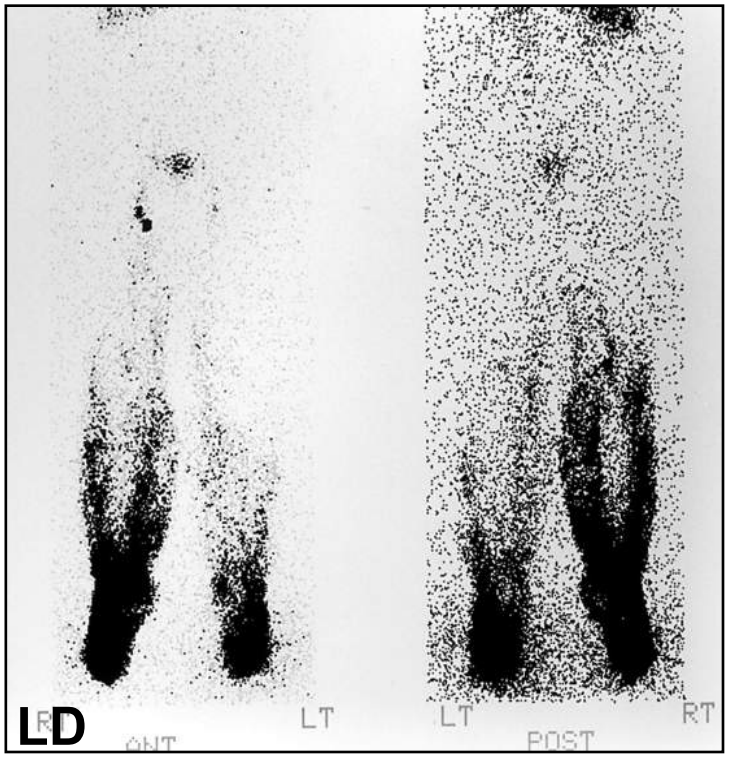
- anomalous pulmonary venous connections
- patent ductus arteriosus

Johnson et al., 1999, Arch. Dermatol.

Lymphatic vessels in LD



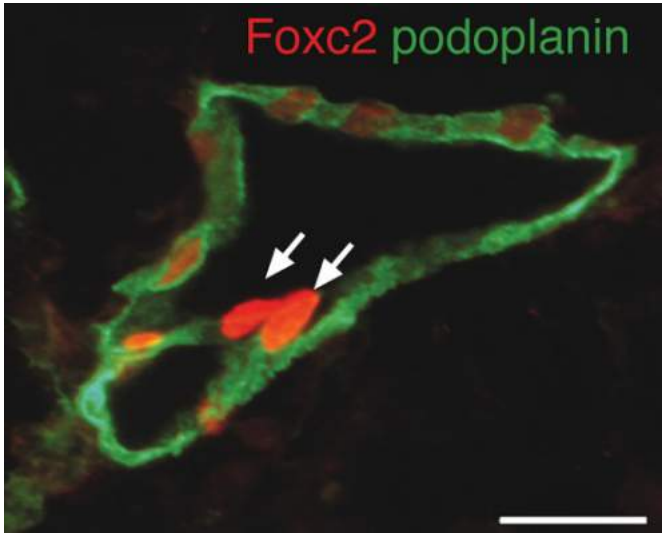


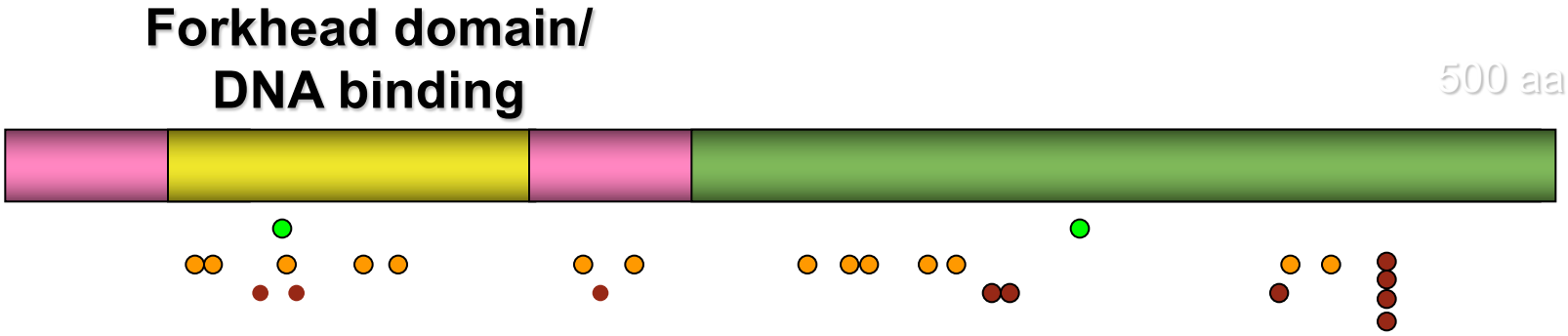


- Normal (Brice et al., 2002) or hyperplastic (Johnson et al, 1999)
- Lymphoscintigram shows reflux of tracer (Brice et al., 2002)
- Lymph node hyperplasia

Forkhead transcription factor FOXC2

- Highly conserved 100 aa fork head DNA binding domain
- Expressed in lymphatic EC, especially in lymphatic valves
- Multiple mutations cause lymphedema-distichiasis





- Fang *et al*., 2000
- Bell *et al*., 2000
- Finegold *et al*., 2001

Main questions

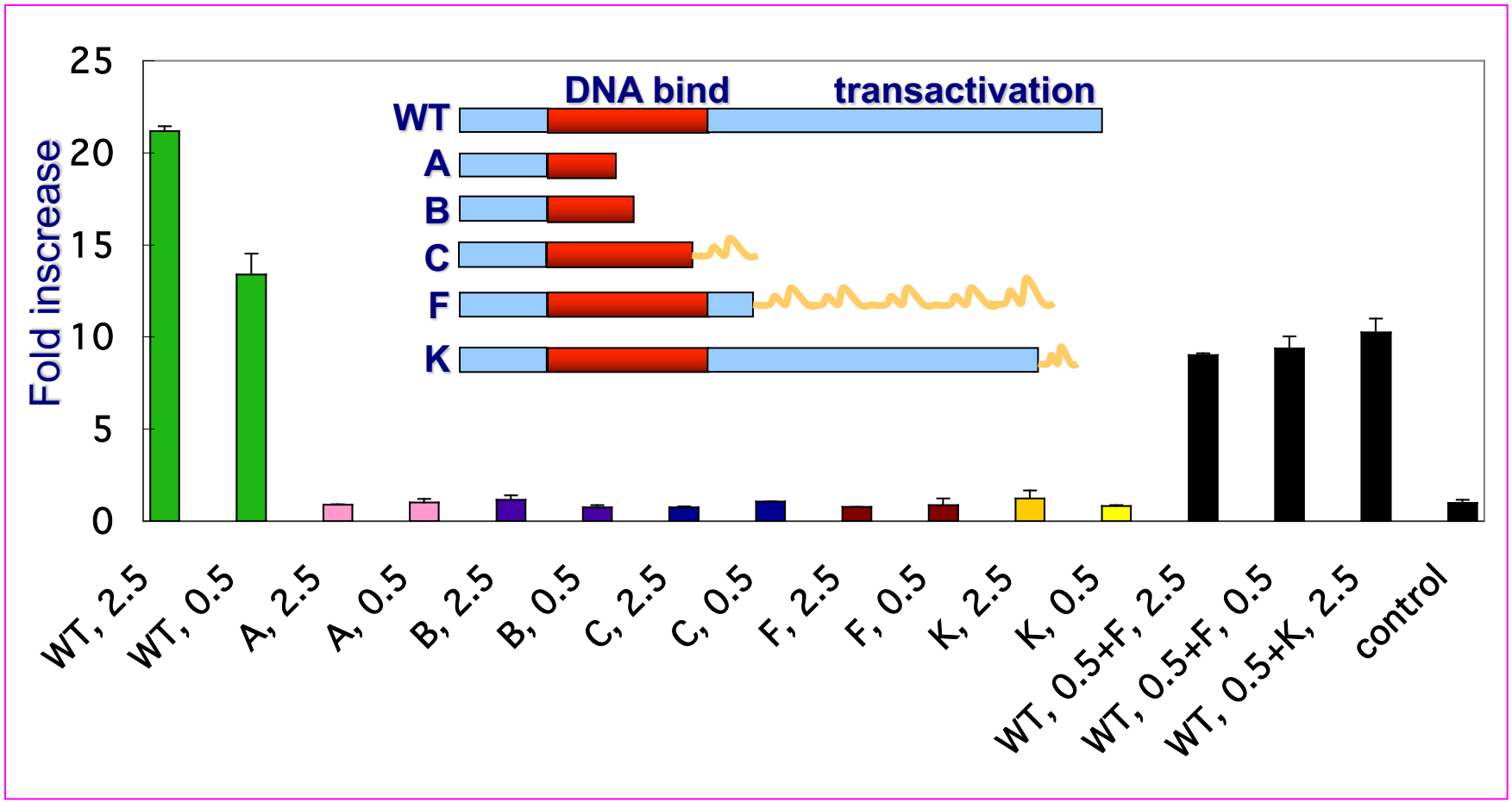
- What is the role of FOXC2 in lymphatic vasculature?

- What are the mechanisms underlying the lymphatic vessel dysfunction in LD?

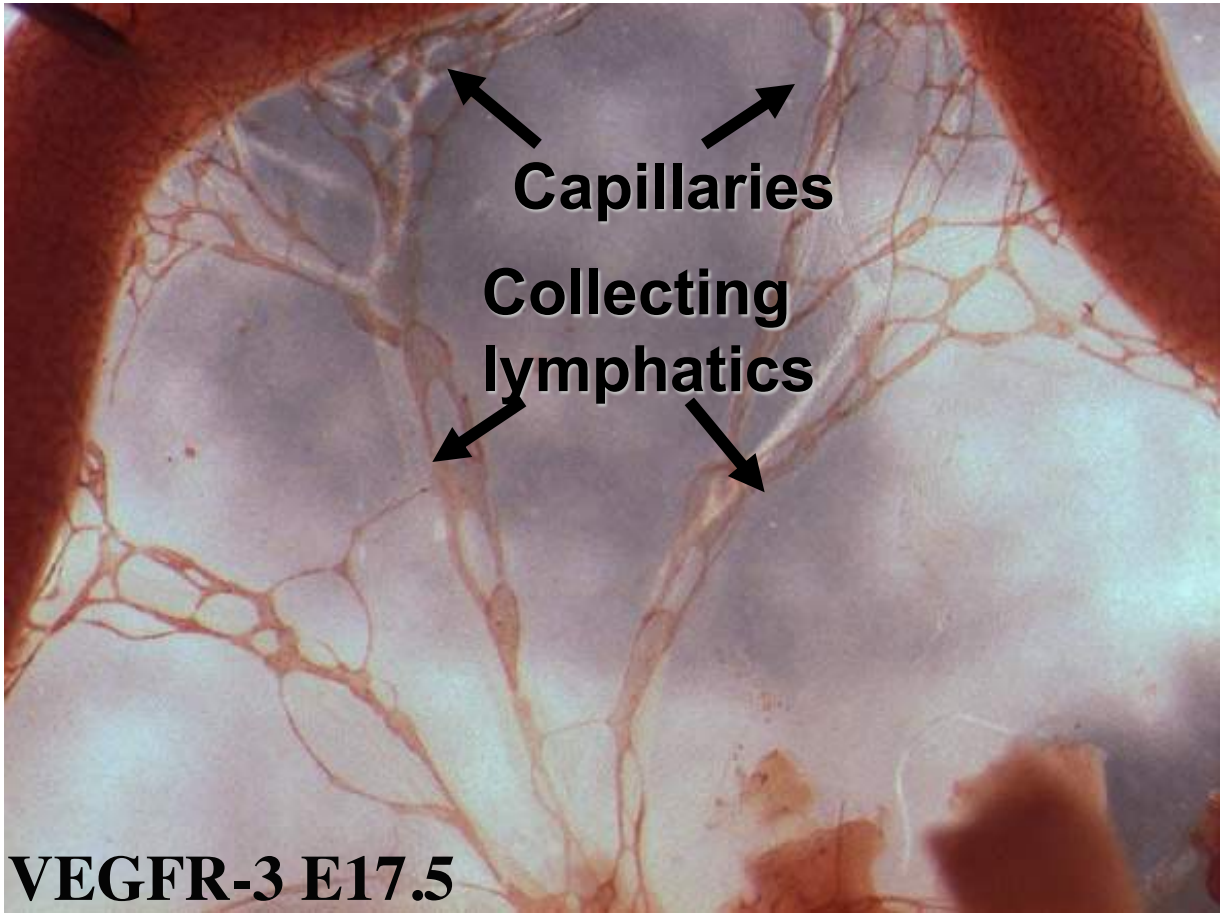
- Can such mechanisms be targeted to improve the lymphatic transport in LD patients?

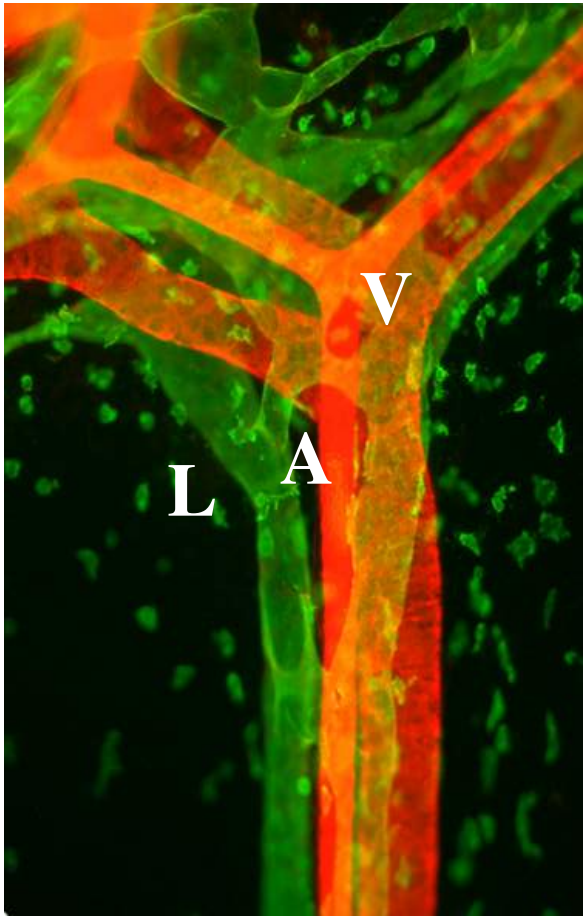
FOXC2 mutants are transcriptionally inactive

Reporter: 6xFOXC2-luci, 2.5 and 0.5 ug of expression constructs



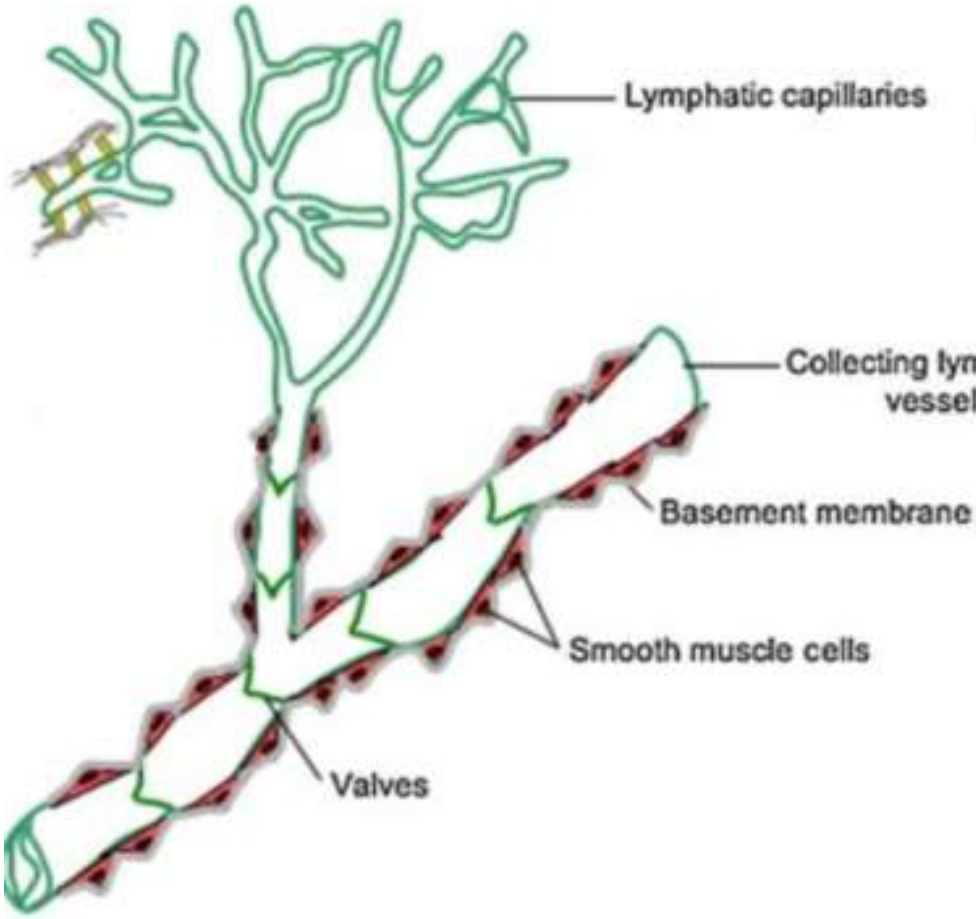
Mouse mesenteric lymphatic vessels as a model





- Thin 2D-like structure
- Stereotypic location of blood and lymphatic vessels
- Both collecting lymphatic vessel and capillaries

Lymphatic vasculature contains two types of vessels



Lymphatic capillaries = fluid/proteins/cells uptake

Collecting vessels = lymph transport

Lymphatic capillary =
fluid/proteins/cells uptake

Collecting vessels =
lymph transport

Efficient lymph transport
and immune response

What is the role of Foxc2 in the embryonic, postnatal and adult lymphatic vasculature?

Embryo

Foxc2$^{-/-}$
Foxc2$^{fl/fl}$; *Prox1-Cre*^{ERT2} mouse models

Postnatal

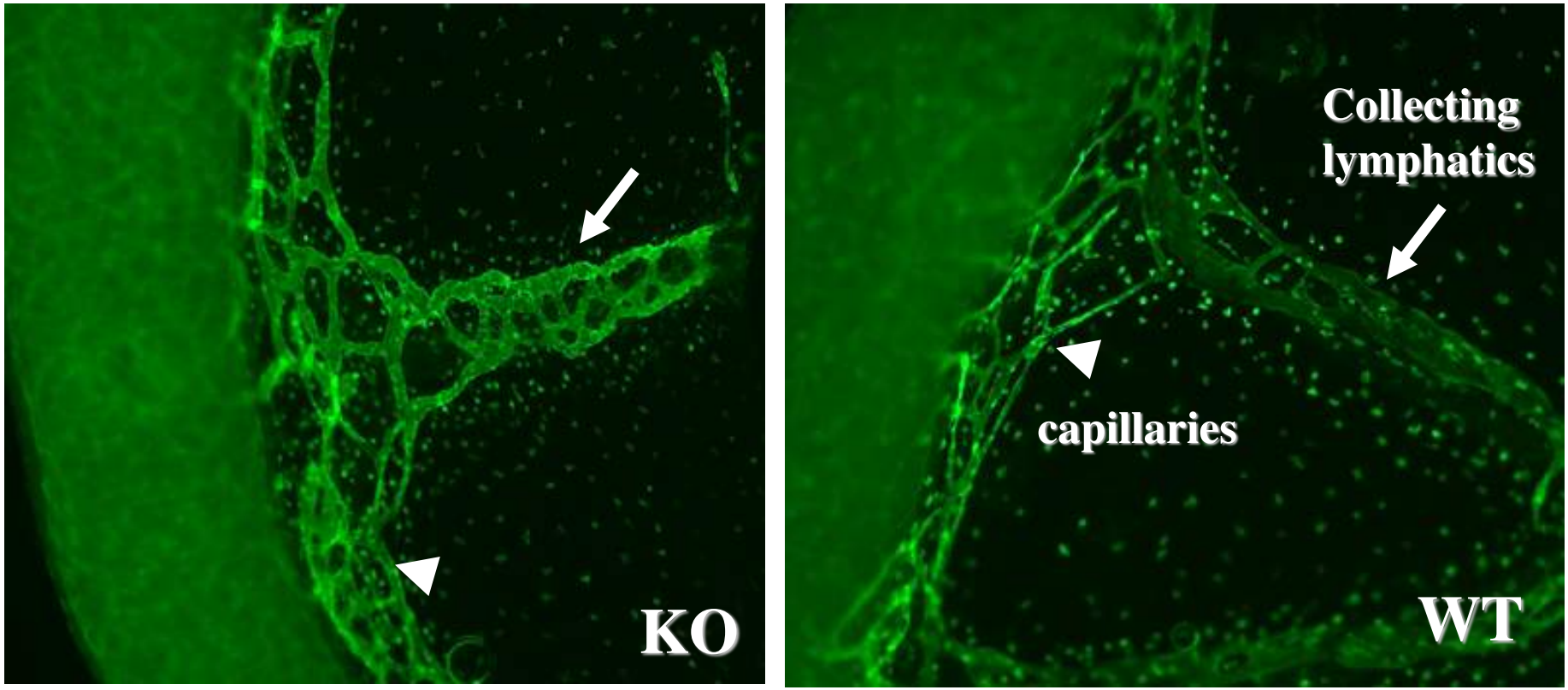
Embryo

Adult

Foxc2$^{fl/fl}$; *Prox1-Cre*^{ERT2} mouse model

Petrova et al., Nat Med 2004
Norrmen et al., J Cell Biol 2009
Sabine et al., Dev Cell., 2012
Sabine et al, *JCI*, 2015

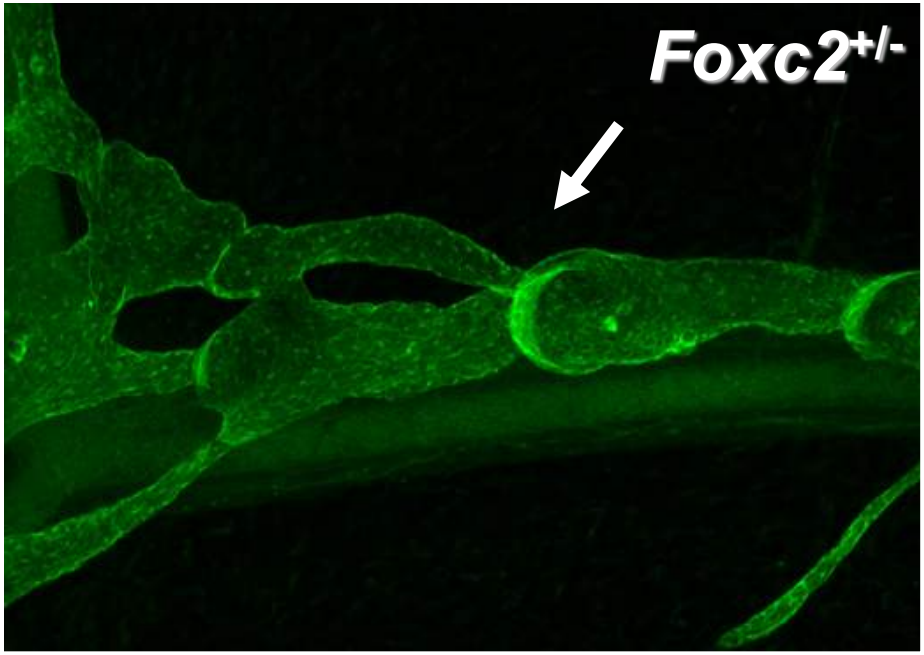
Foxc2 controls formation of collecting lymphatic vessels during embryogenesis

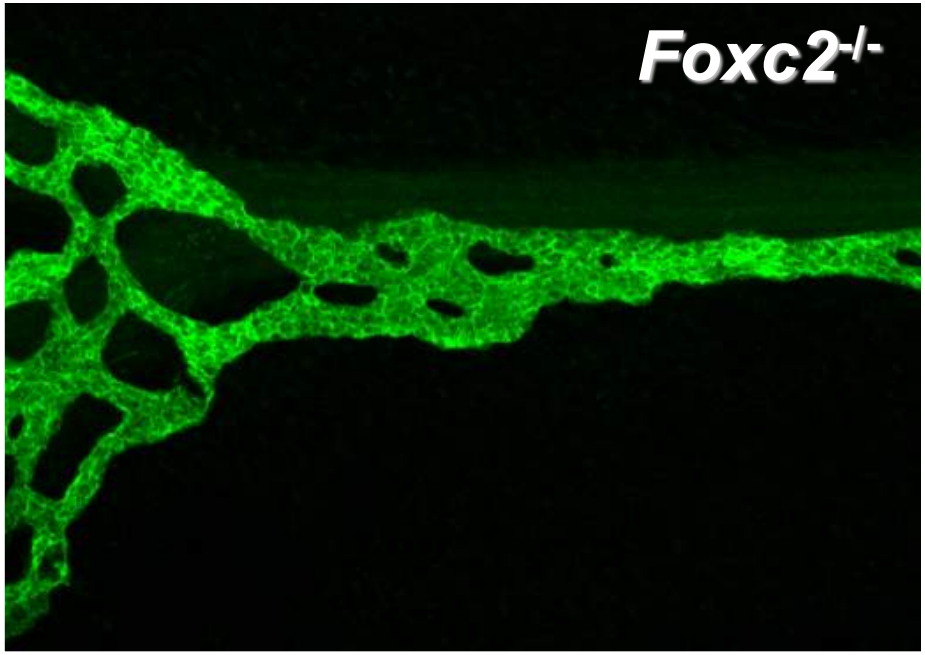


LYVE-1

E17.5 mesenteric vessels

Agenesis of lymphatic valves in *Foxc2* germline knockout mice





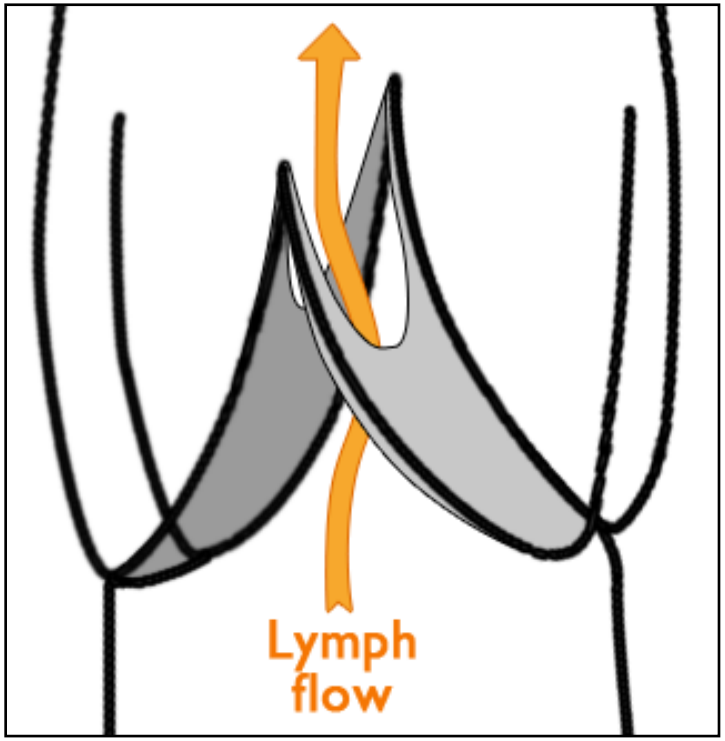
VEGFR-3

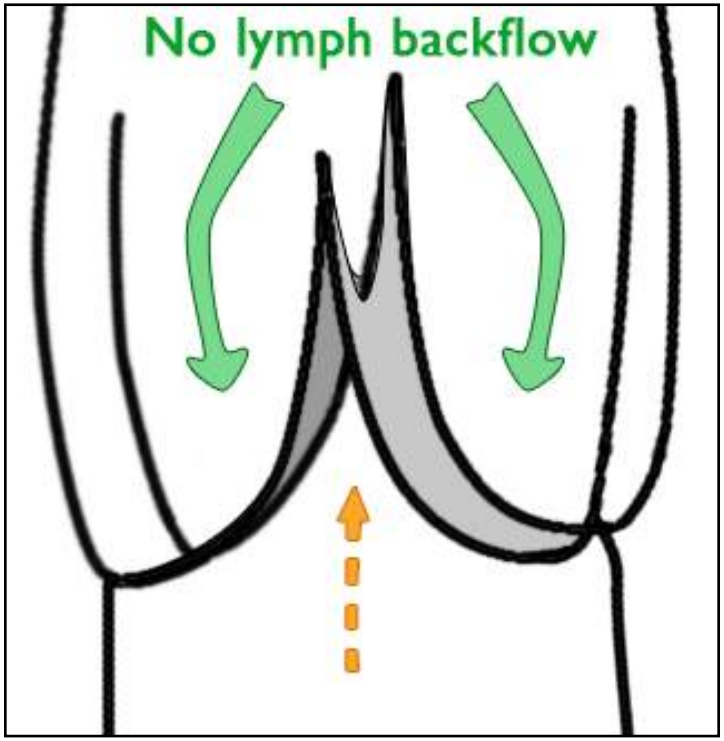
E17.5 mesenteric vessels

Petrova et al., Nat Med, 2004
Norrmén et al., J. Cell Biol. 2009

Lymphatic valves prevent lymph backflow

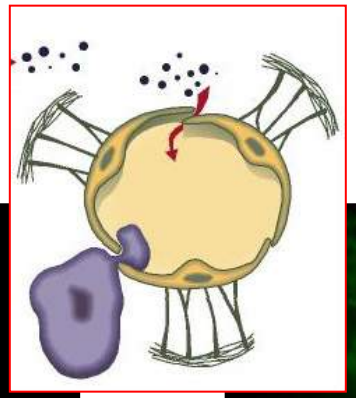
Unidirectional movement of lymph

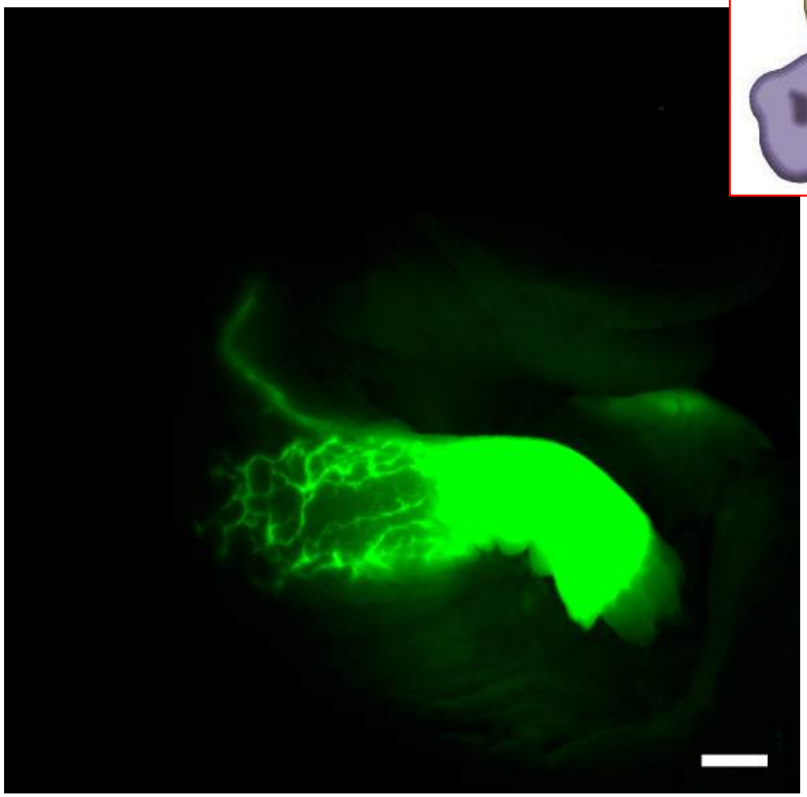


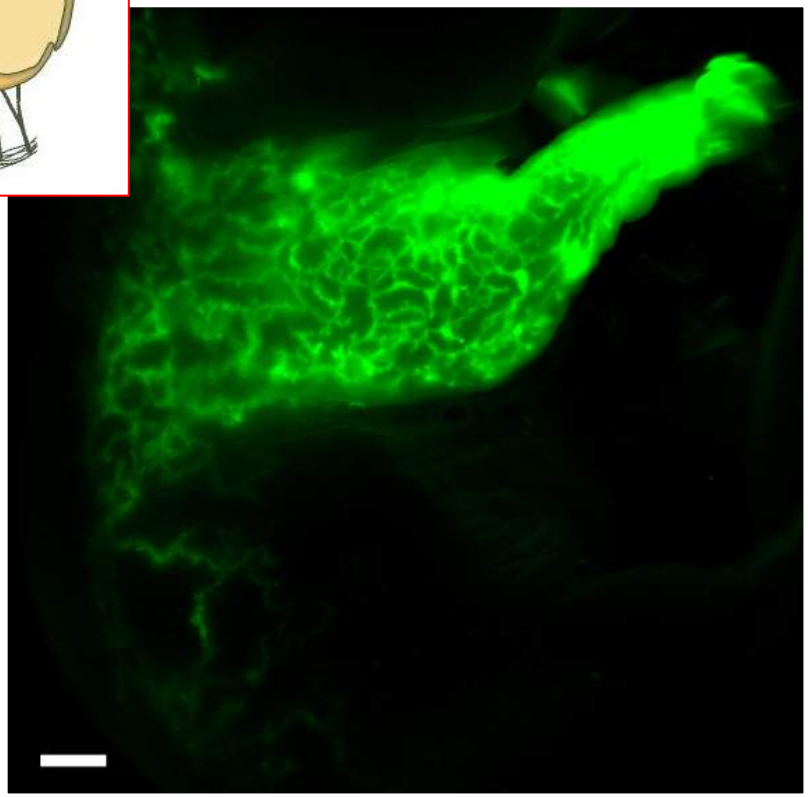


Agenesis of lymphatic valves leads to inefficient lymph transport

FITC-dextran



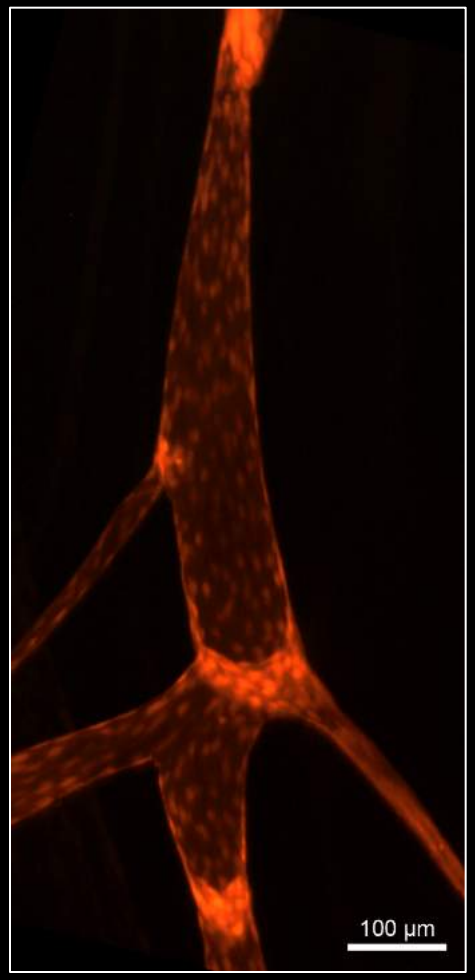


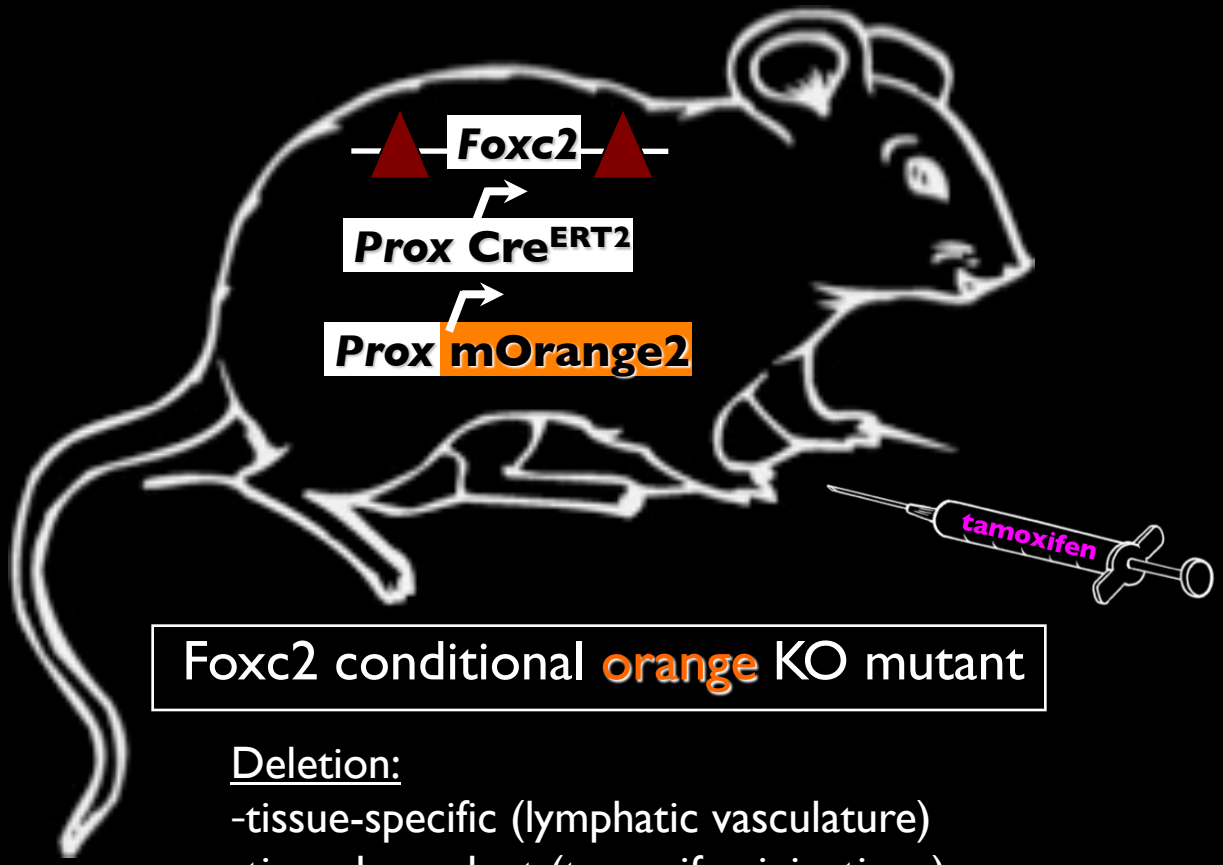


WT

Foxc2^{lecKO}

Is Foxc2 important for collecting lymphatic vessel maintenance?



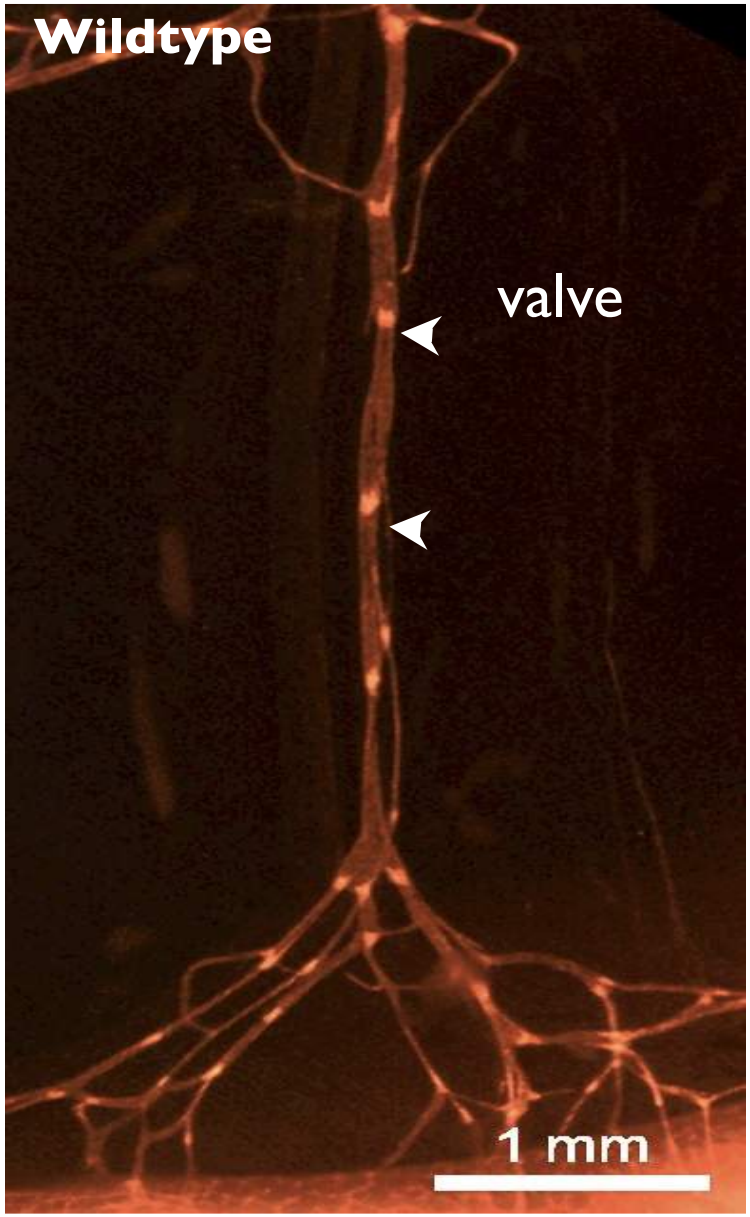


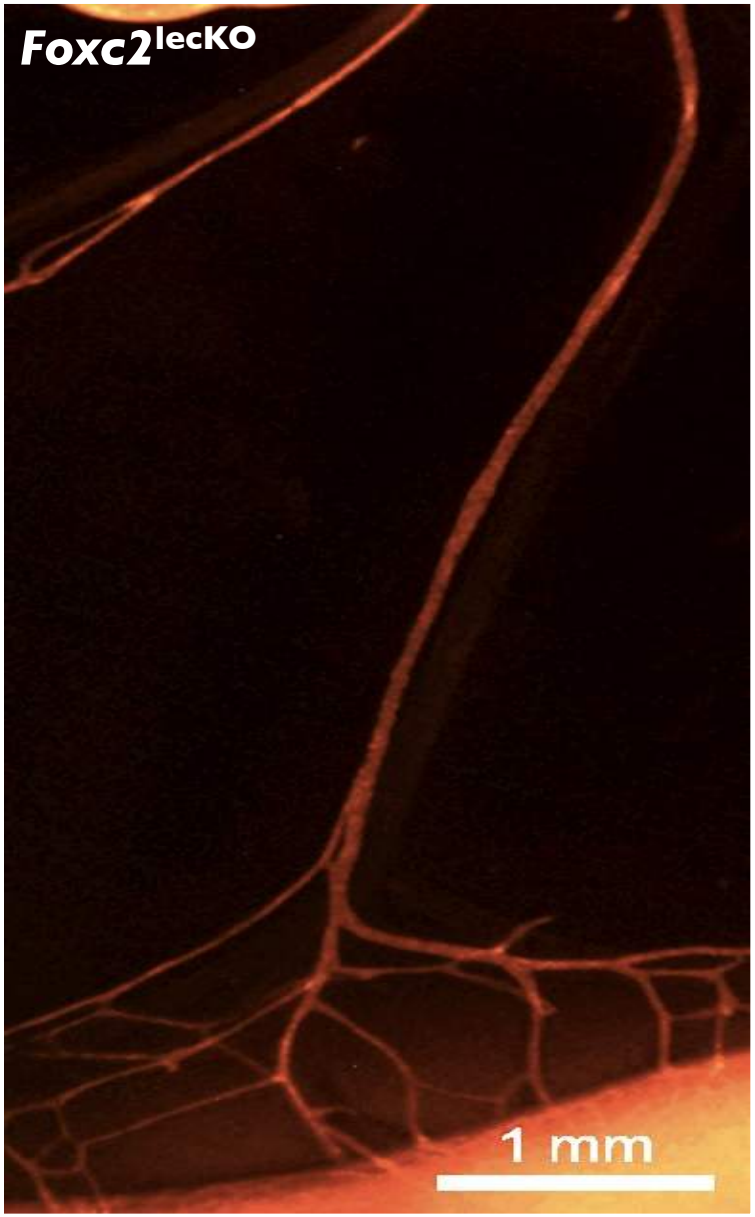
Foxc2 conditional orange KO mutant

Deletion:
-tissue-specific (lymphatic vasculature)
-time-dependent (tamoxifen injections)
-expression of an orange lymphatic marker

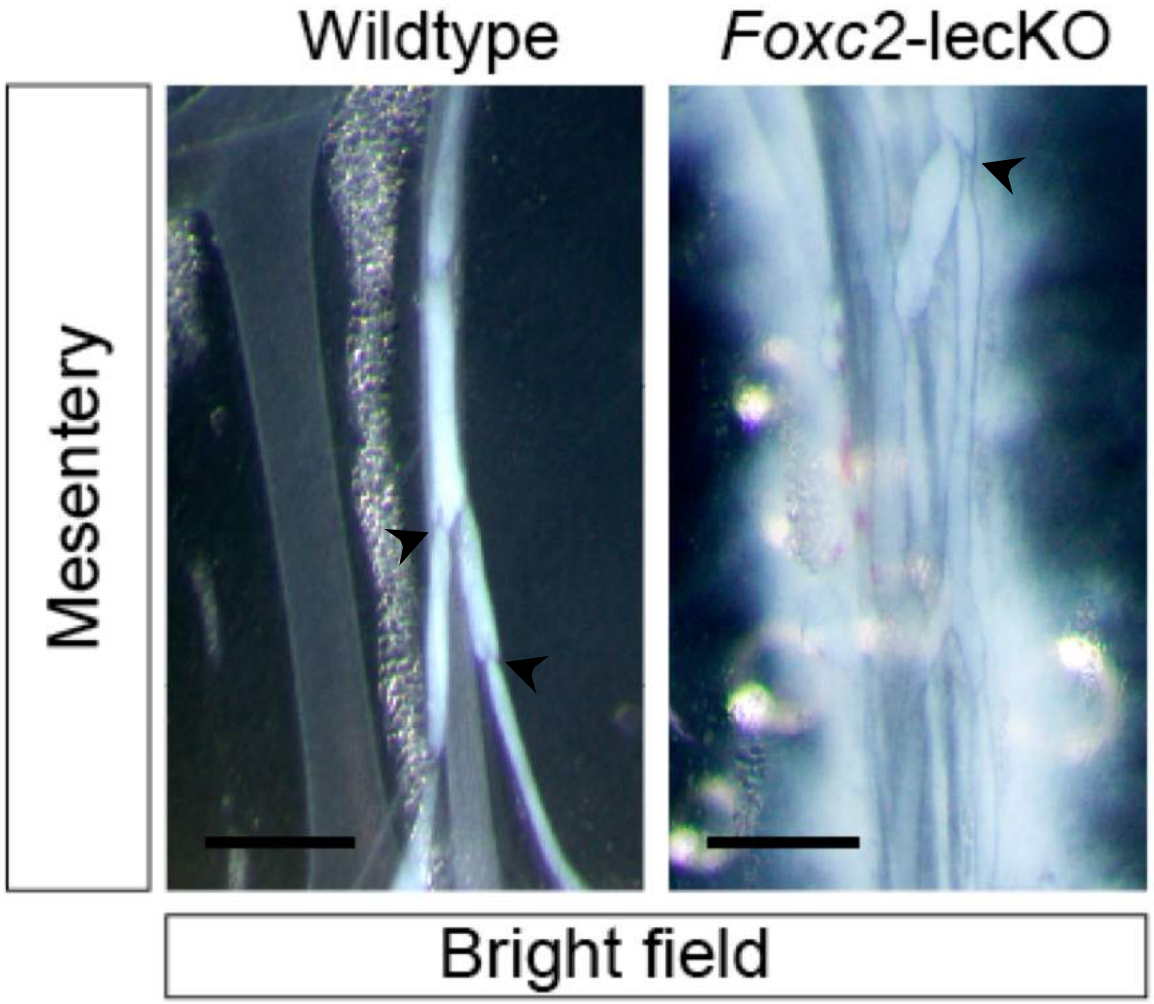
***Prox1*-mOrange2**: Friedemann Kiefer
***Prox1*-CreERT2:** Tajia Makinen

Degeneration of lymphatic valves in Foxc2^{lecko} mice

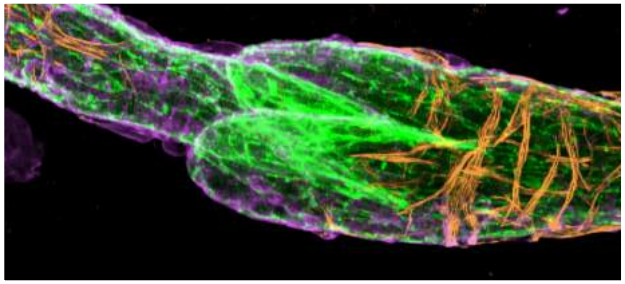




Foxc2 postnatal deletion induces leaky collecting vessels



? → **Foxc2** ↔ ?

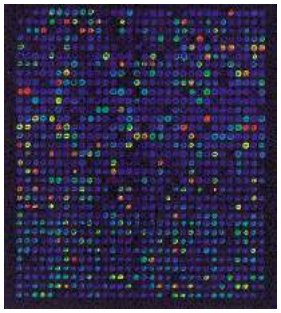


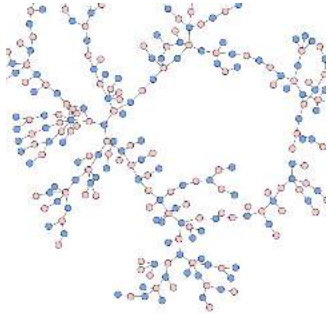
Formation and maintenance of collecting lymphatic vessels and valves

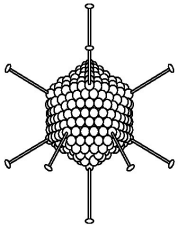
↓

Directional transport of lymph and impermeability of collecting vessels

Analysis of FOXC2 transcriptional network in lymphatic endothelial cells







Primary lymphatic endothelial cells

siRNA knockdown
FOXC2 overexpression

Chromatin
immunoprecipitation

Transcriptome
profiling

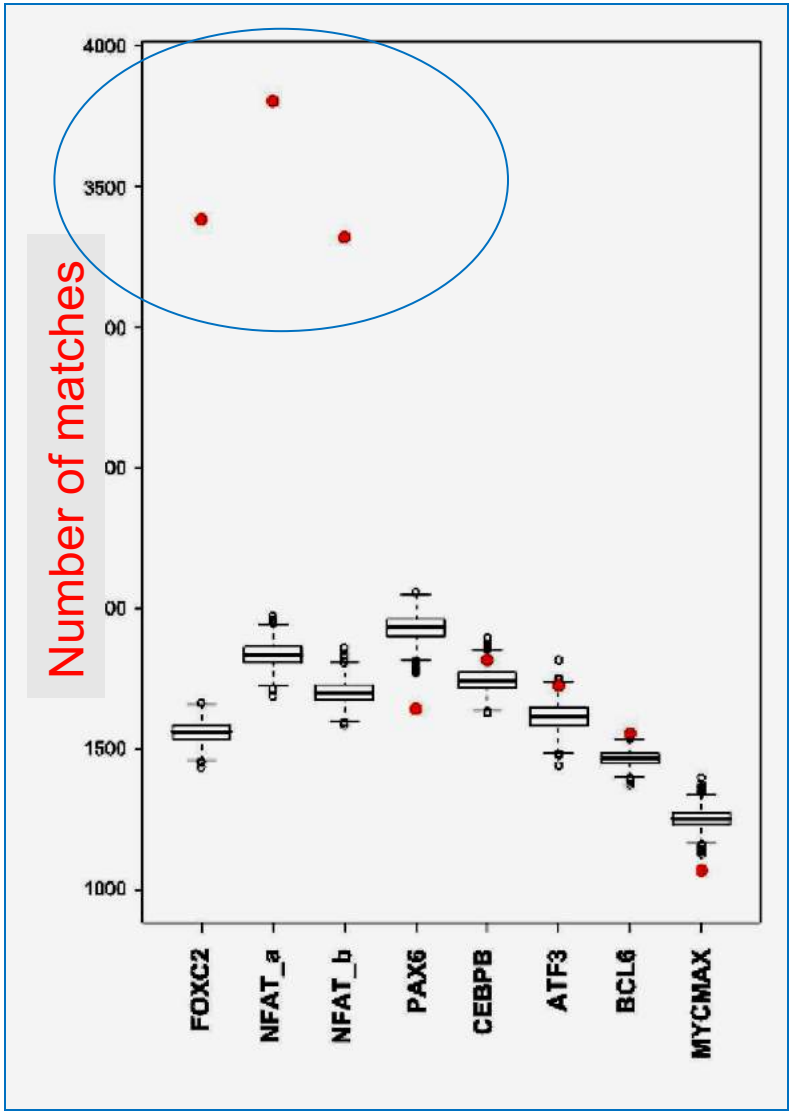
High throughput mapping
of FOXC2-DNA
interactions

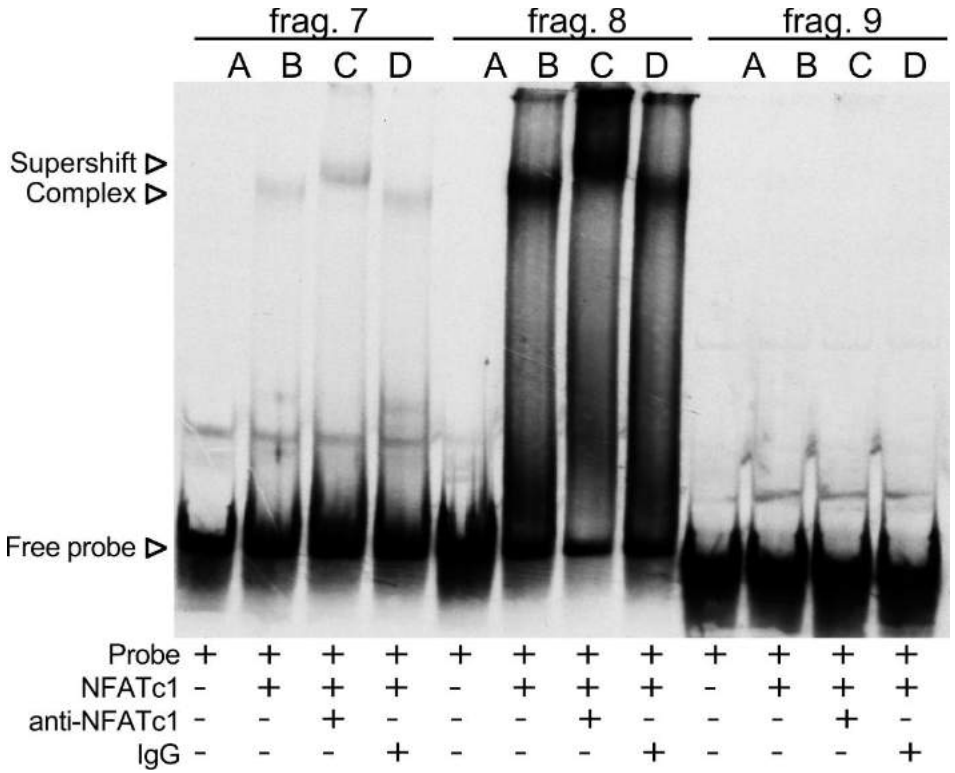
Bioinformatics analysis

FOXC2 target genes

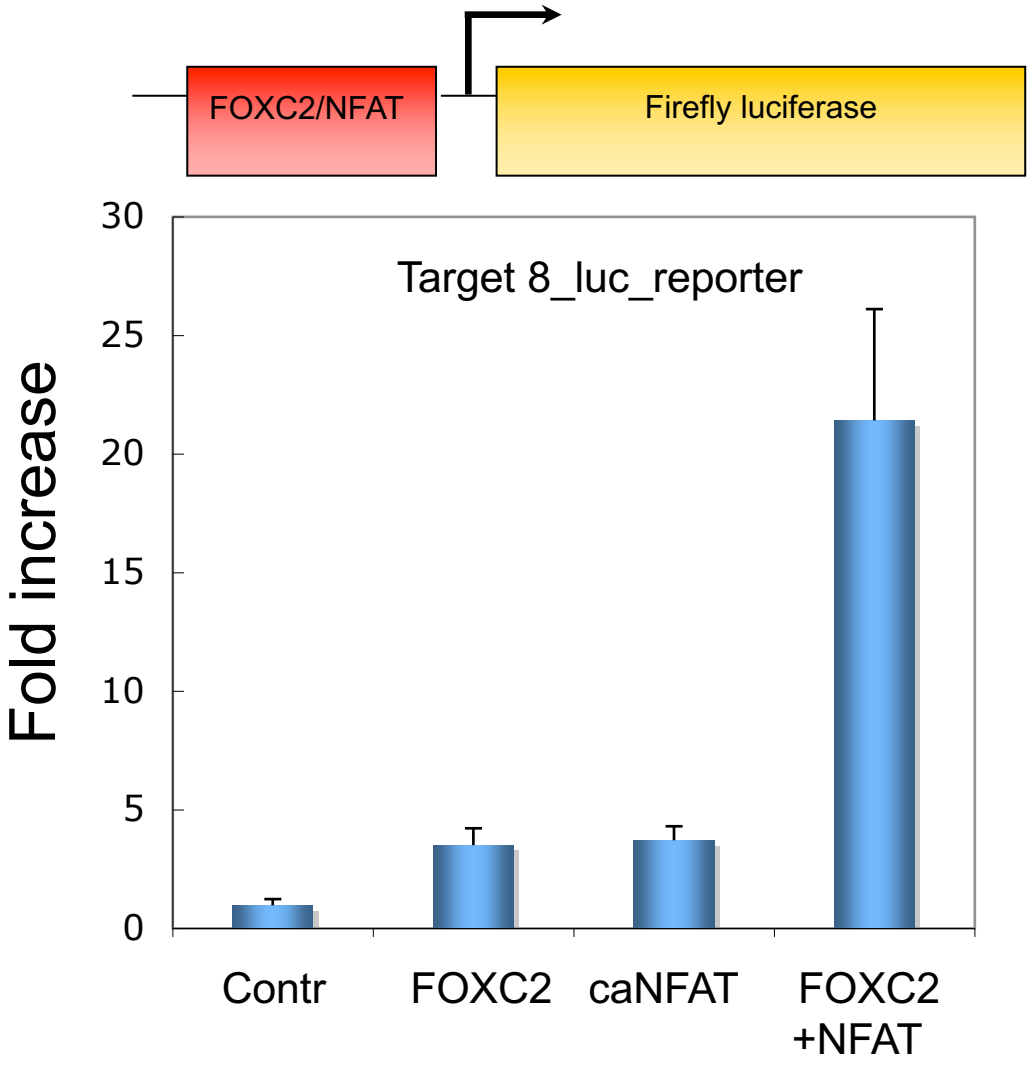
Novel regulators of lymphatic vascular development

FOXC2 bound enhancers are enriched in NFAT sites



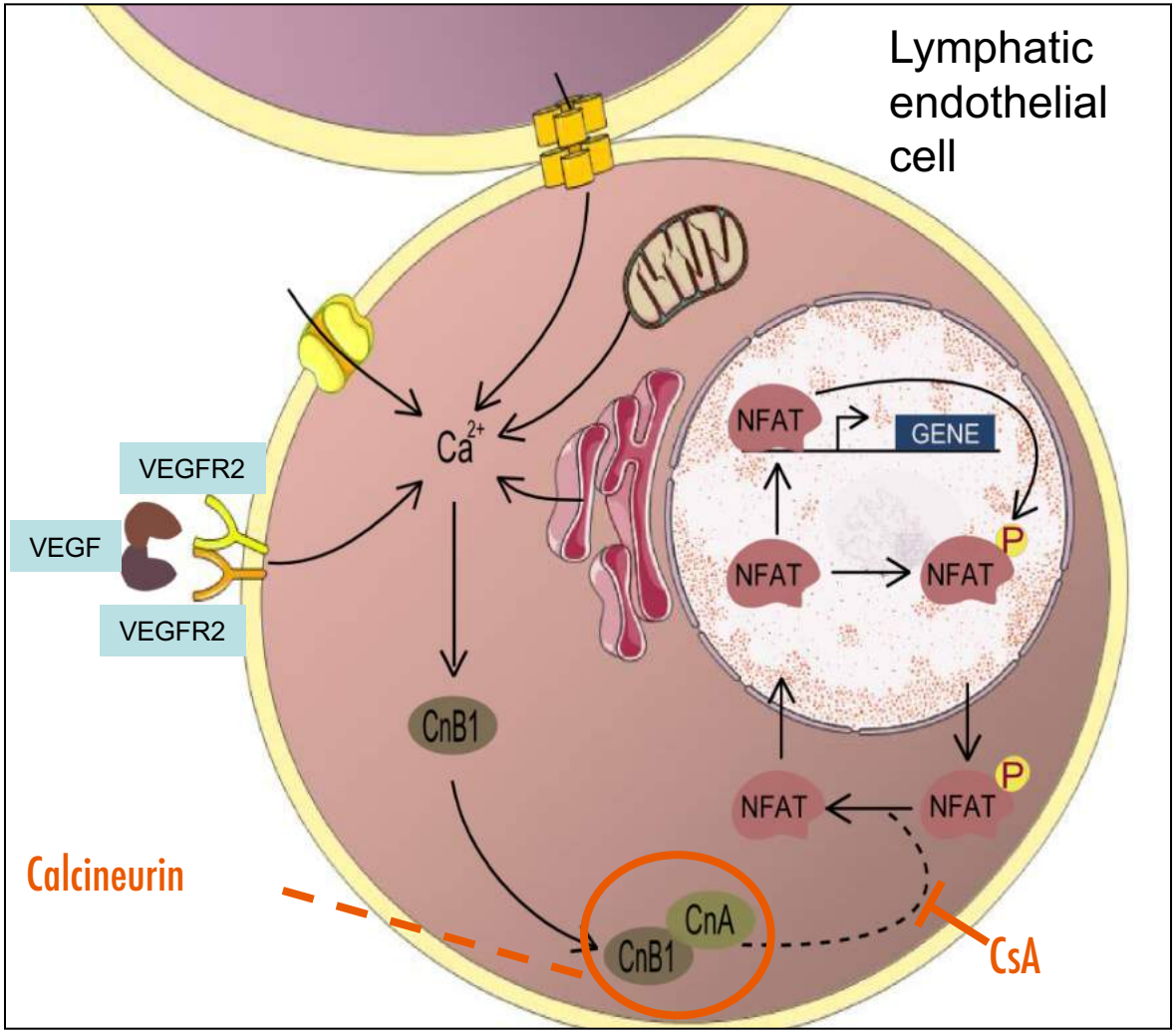


FOXC2 and NFAT cooperate on composite enhancers



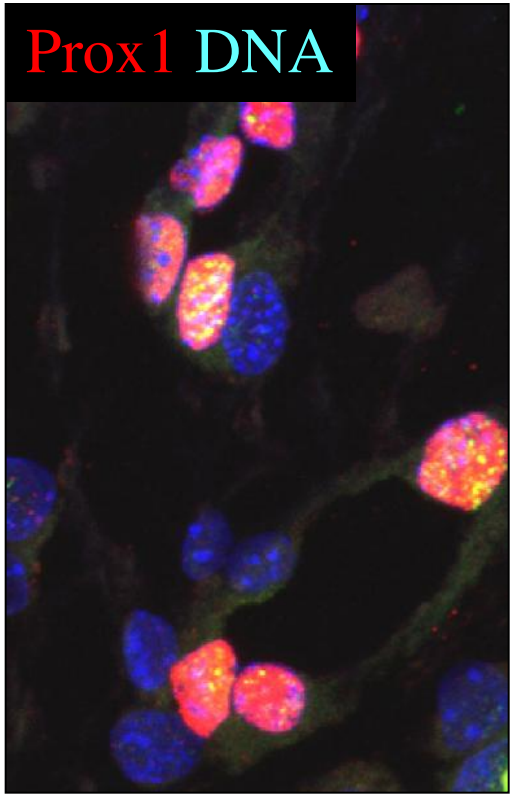
Norrmèn et al. J. Cell Biol., 2009

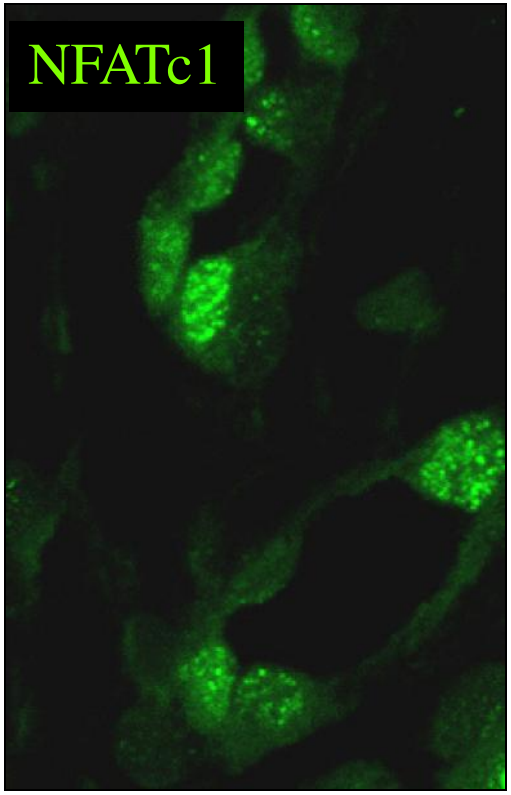
Calcineurin/NFAT pathway

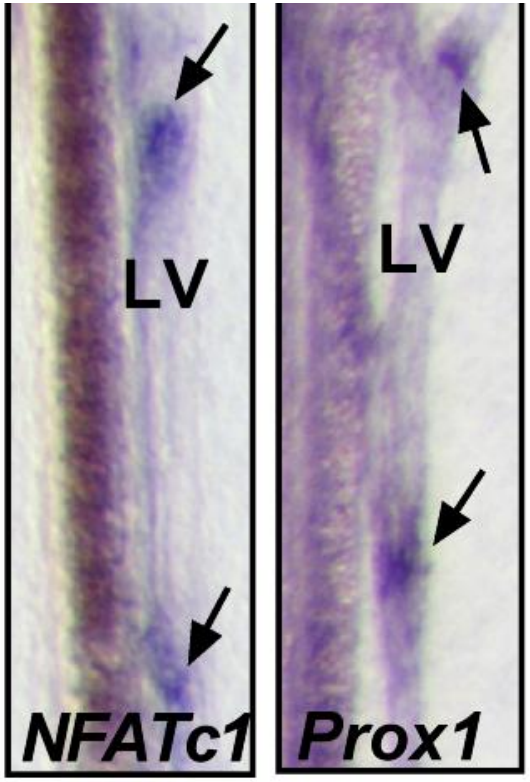


- important in lymphocytes (IL-2), cardiac, skeletal and smooth muscle cells
- control of pro-inflammatory genes in blood endothelial cells downstream of VEGF/VEGFR-2
- role in lymphatic vasculature?

NFATc1 in lymphatic vessels: nuclear localization and high expression in valves



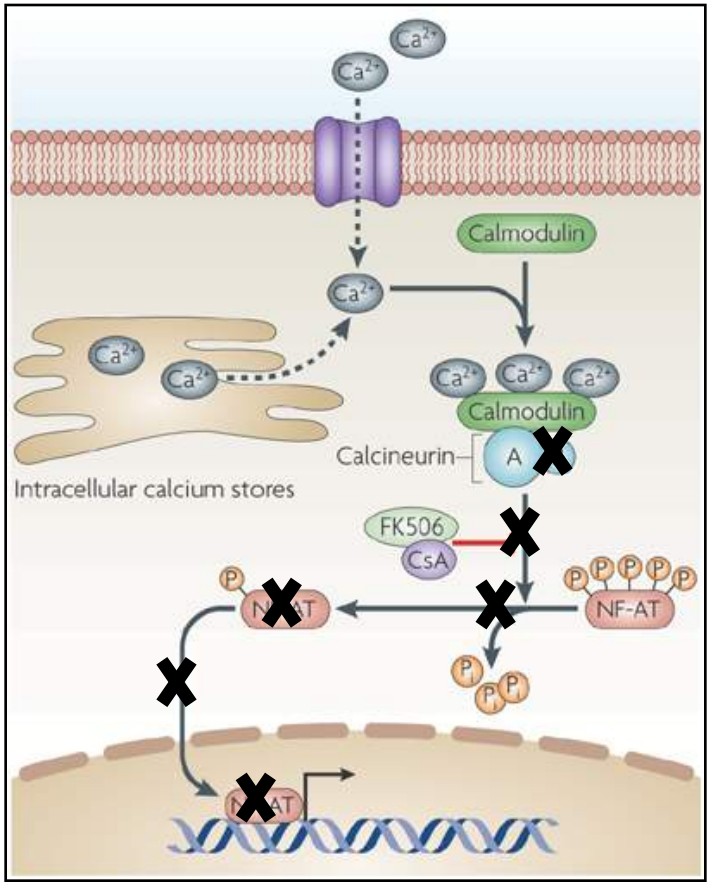




LV=lymphatic vessel

Genetic analysis of calcineurin/NFAT signaling in lymphatic vascular development

Amelie
Sabine

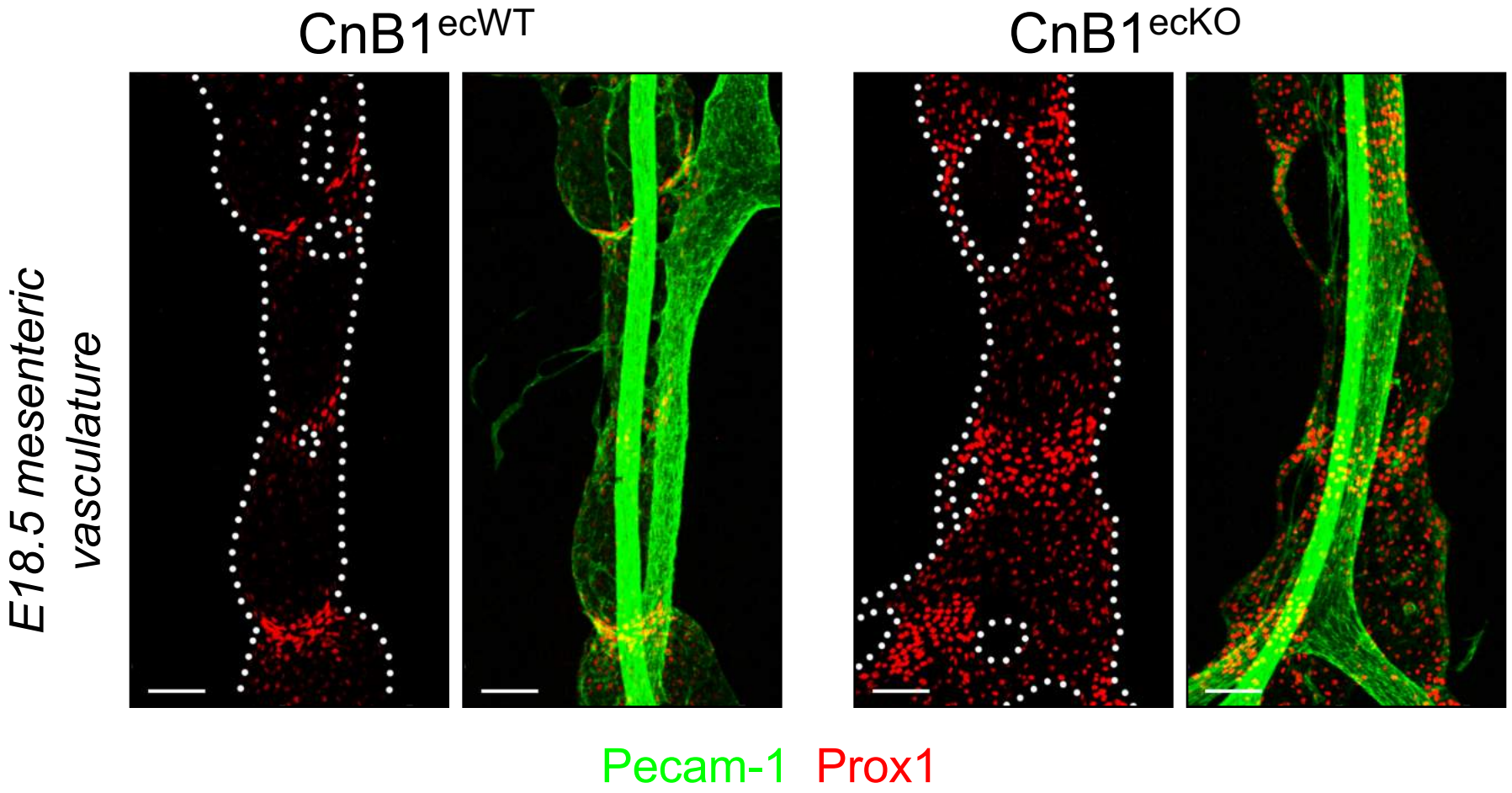


x

Tamoxifen-inducible inactivation of CnB1:

- PDGFβ-iCre^{ERT2} ➳ blood endothelium
- VEC-Cre^{ERT2} ➳ blood and lymphatic endothelium
- Prox1-Cre^{ERT2} ➳ lymphatic endothelium

Pan-endothelial or lymphatic endothelial deletion of calcineurin prevents lymphatic valve formation

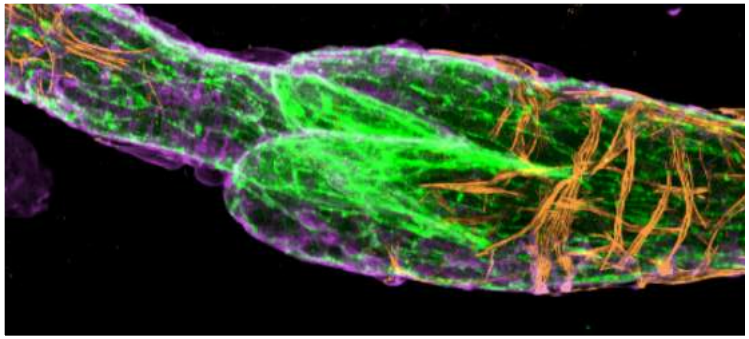


Foxc2

Calcineurin/NFAT

?

Formation and maintenance of collecting lymphatic vessels and valves



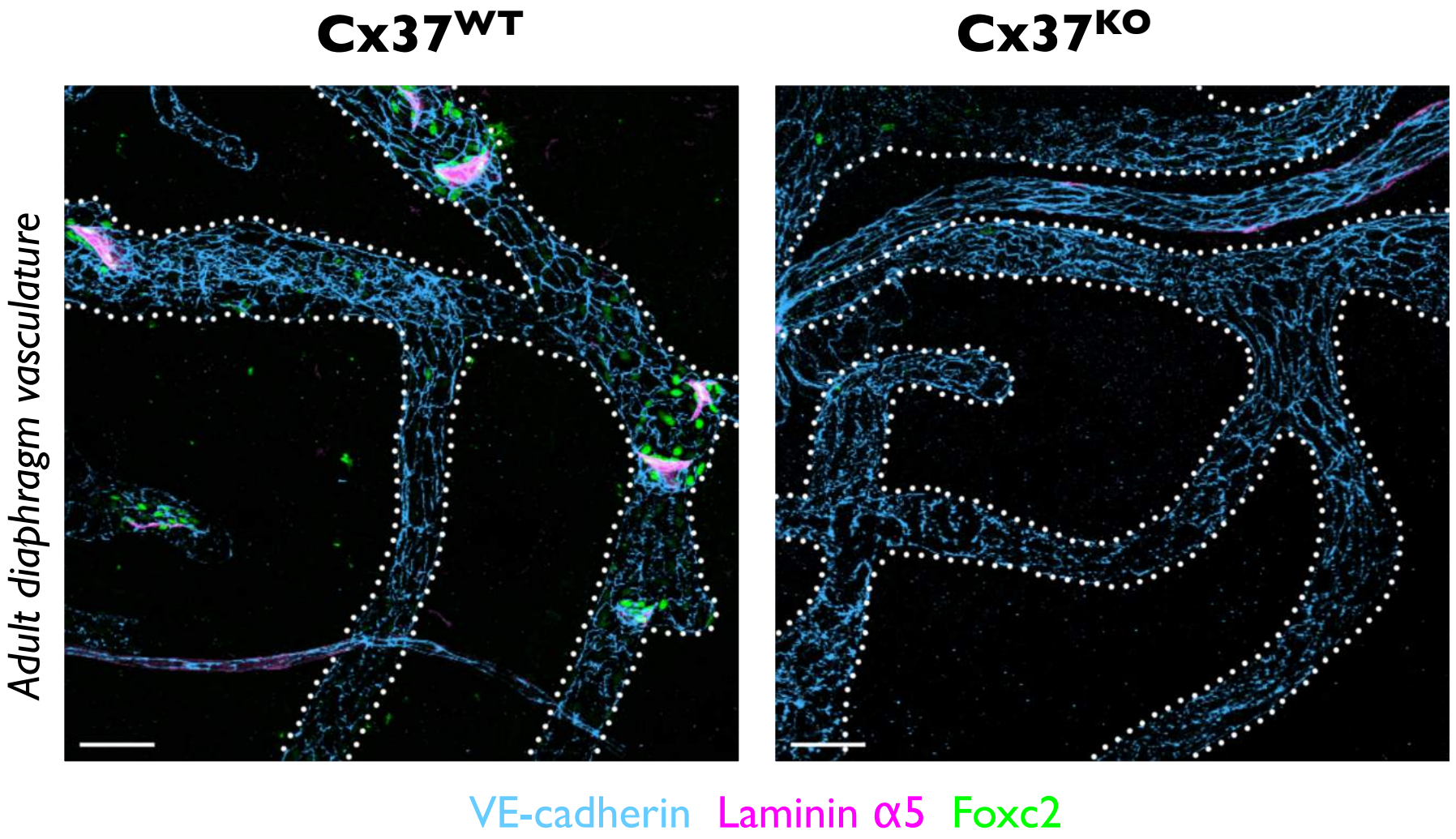
Foxc2

Gene expression analysis
after FOXC2 knockdown *in vitro*

Gene expression analysis from
ex vivo LECs from Foxc2^{KO}

Connexin 37

Cx37$^{-/-}$ adult mice do not have lymphatic valves



Foxc2

↘

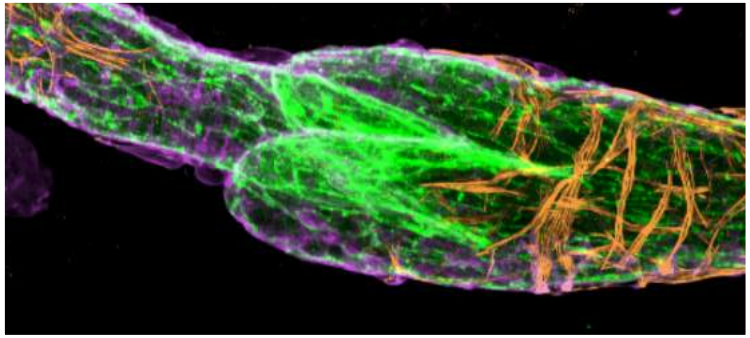
Calcineurin/NFAT

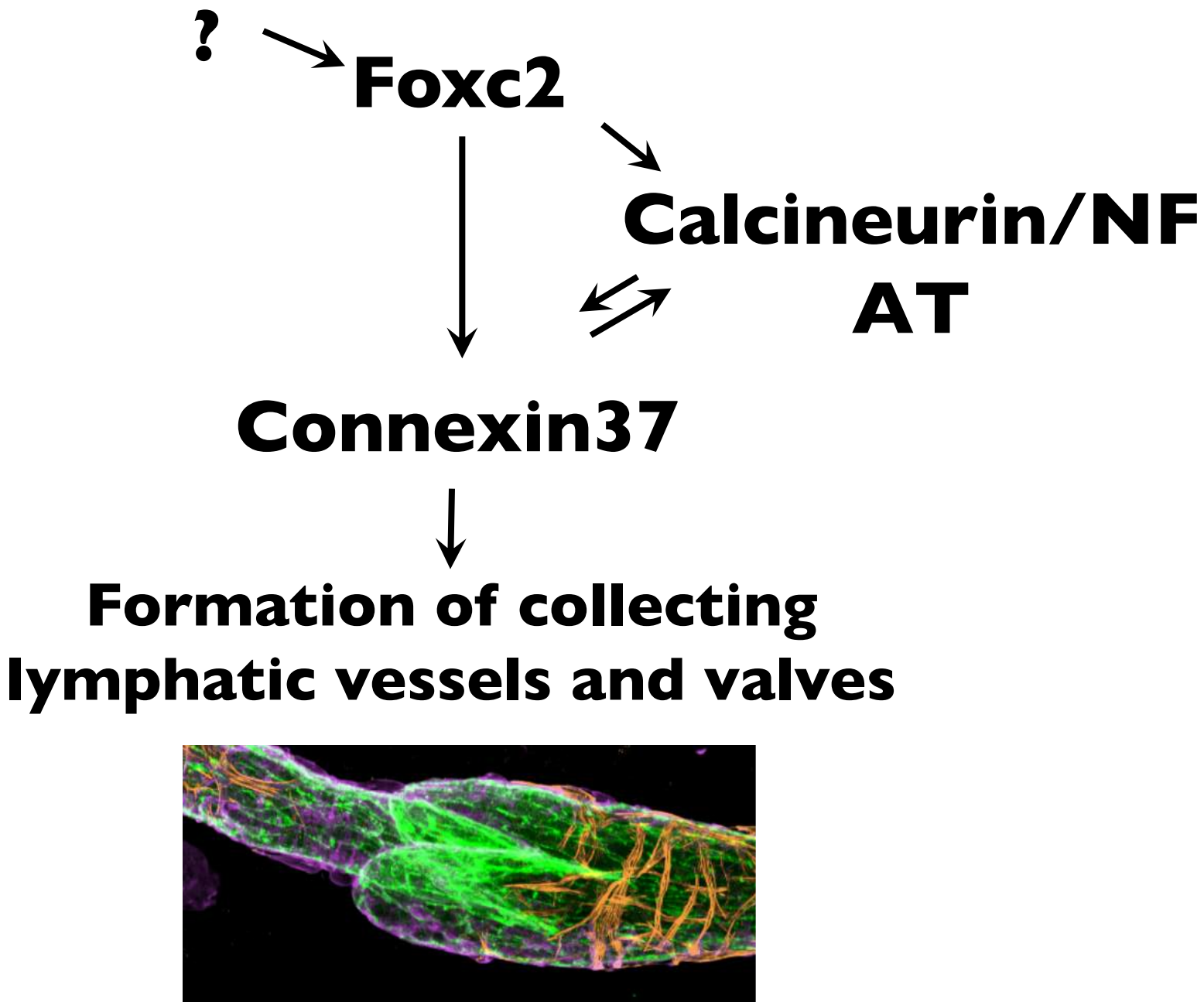
↓ ⇄

Connexin37 (+other targets)

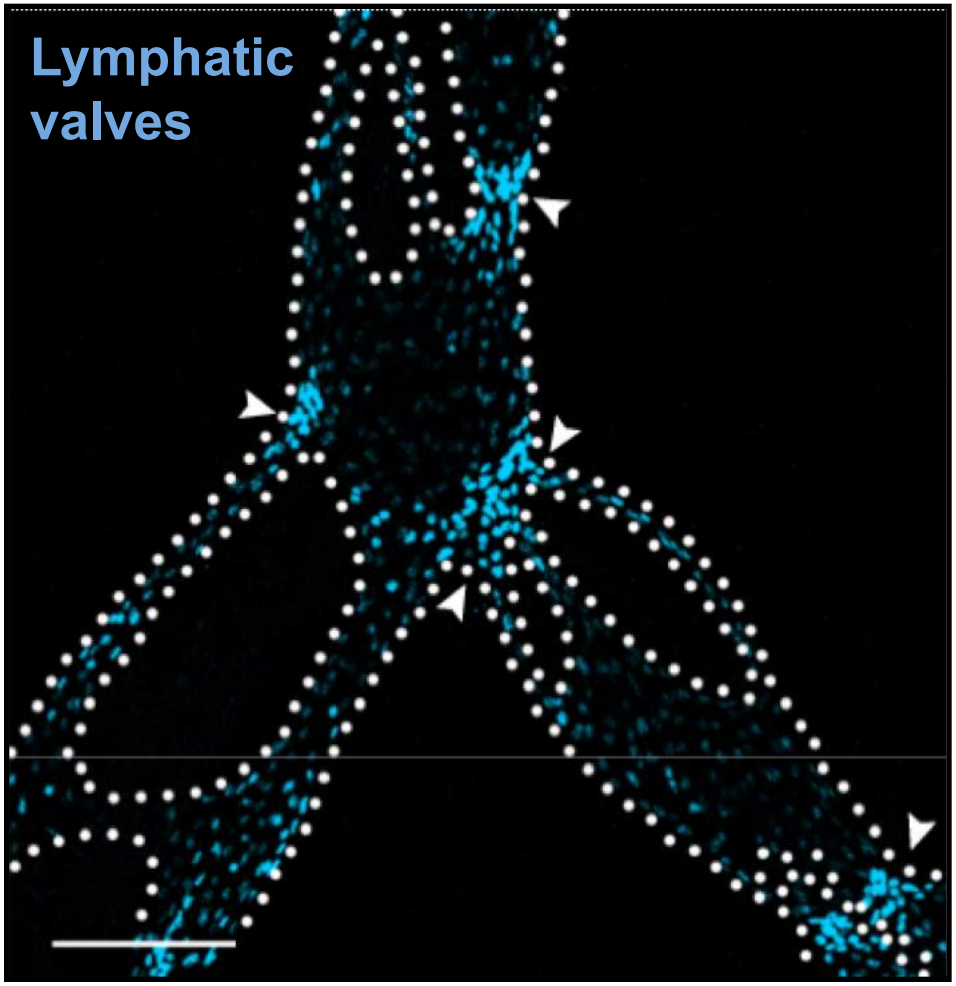
↓

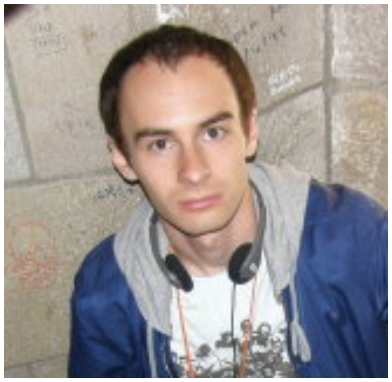
Formation and maintenance of collecting lymphatic vessels and valves




?
Foxc2
Calcineurin/NF
AT
Connexin37
Formation of collecting
lymphatic vessels and valves

Lymphatic valves are frequently located at branching points/bifurcations: role of disturbed flow?

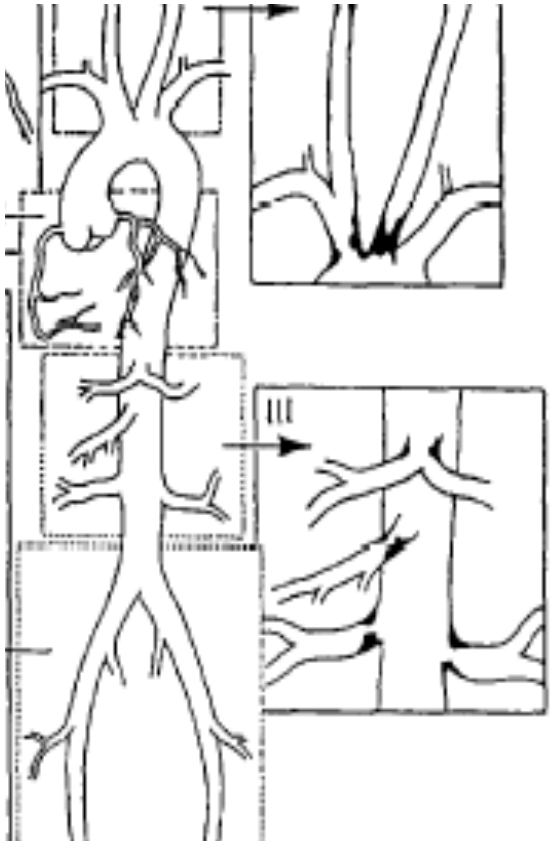




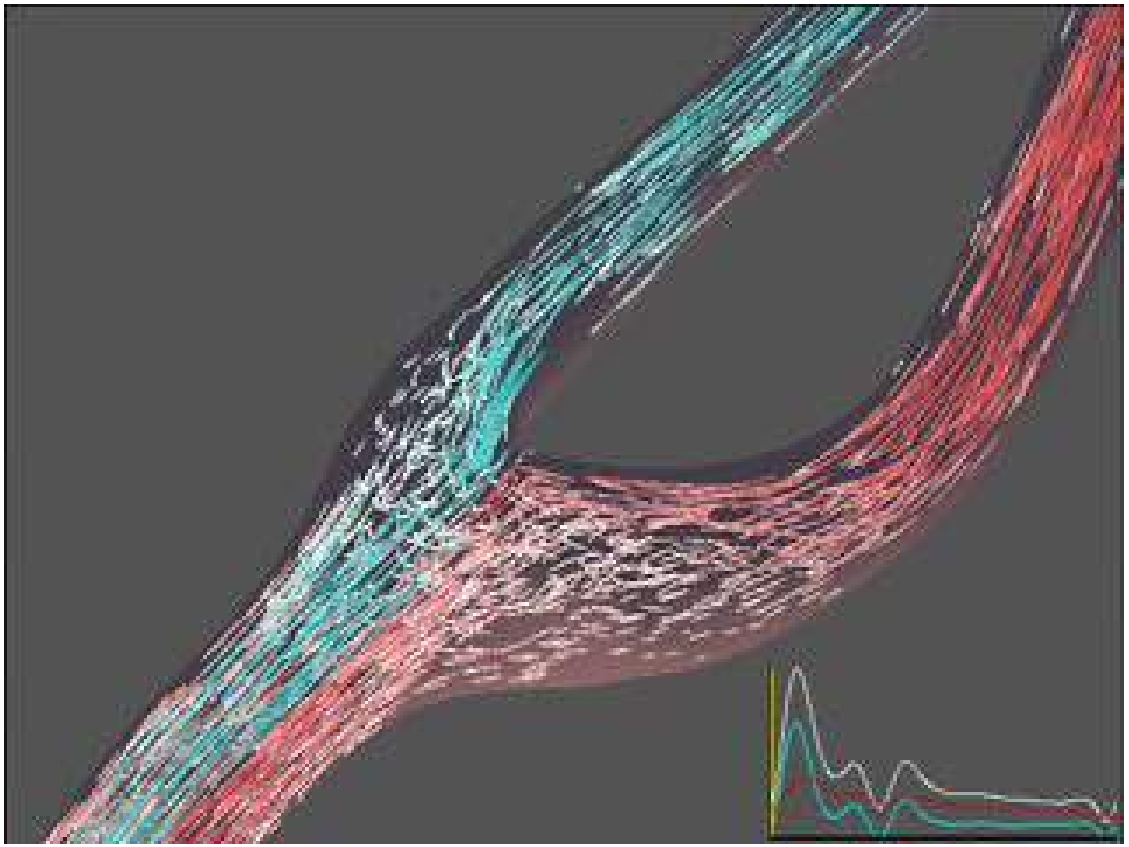
Yan Agalarov

Parallels with atherosclerosis : **role of disturbed flow?**

DeBakey ME *et al.* Annals of Surgery 1985



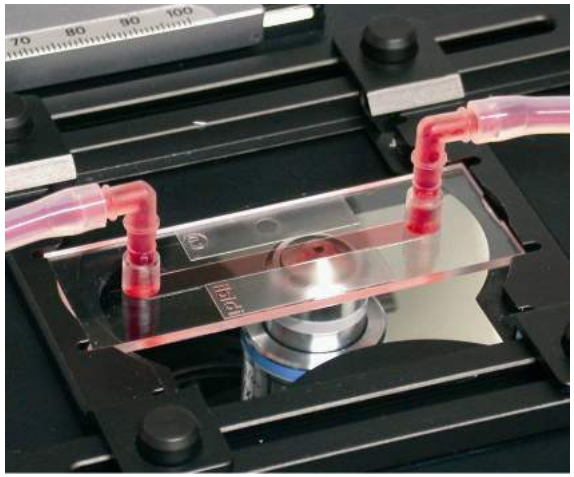
Steinman DA. J. Biomechanics 2000



Predilection sites are: curved vessels, bifurcations at side branches
Low laminar or oscillatory flow induce atherosclerosis

Analysis of LEC mechanosensory responses *in vitro*

Parallel plate flow system	Conditions	Output parameters



Primary human lymphatic endothelal cells

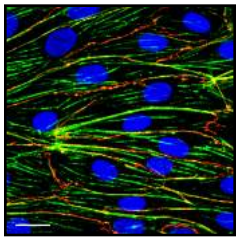
Static

↔

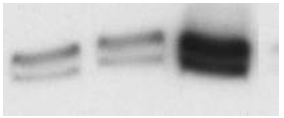
Oscillatory shear stress

→

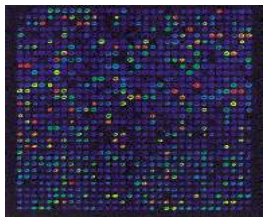
Laminar shear stress



Imaging



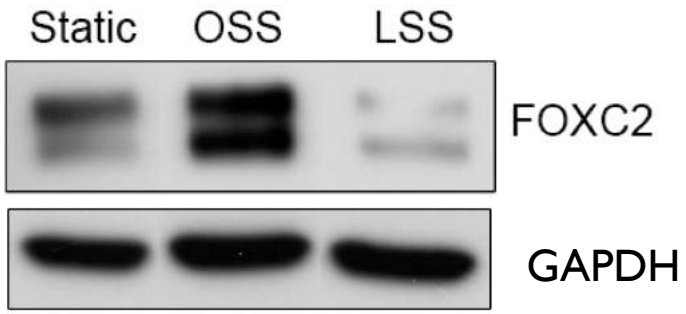
Western



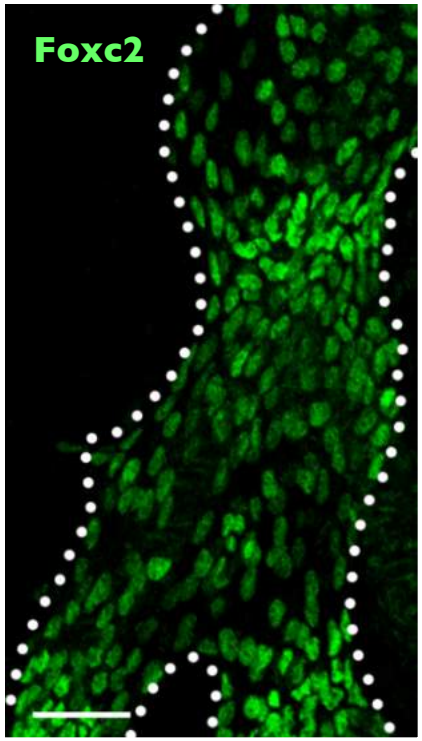
Expression profiles

Reversing flow induces expression of Foxc2

In vitro



In vivo

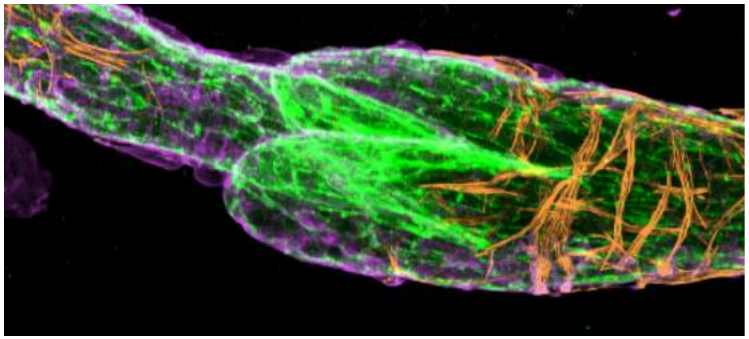


Shear stress → Foxc2

Foxc2 → Calcineurin/NFAT

Foxc2 → Connexin37+other targets

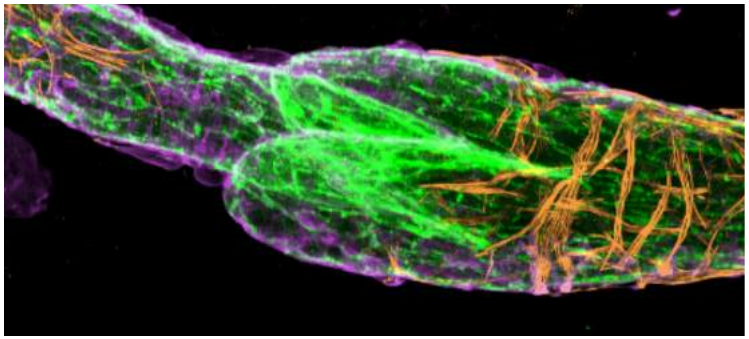
Connexin37+other targets → Formation and maintenance of collecting lymphatic vessels and valves



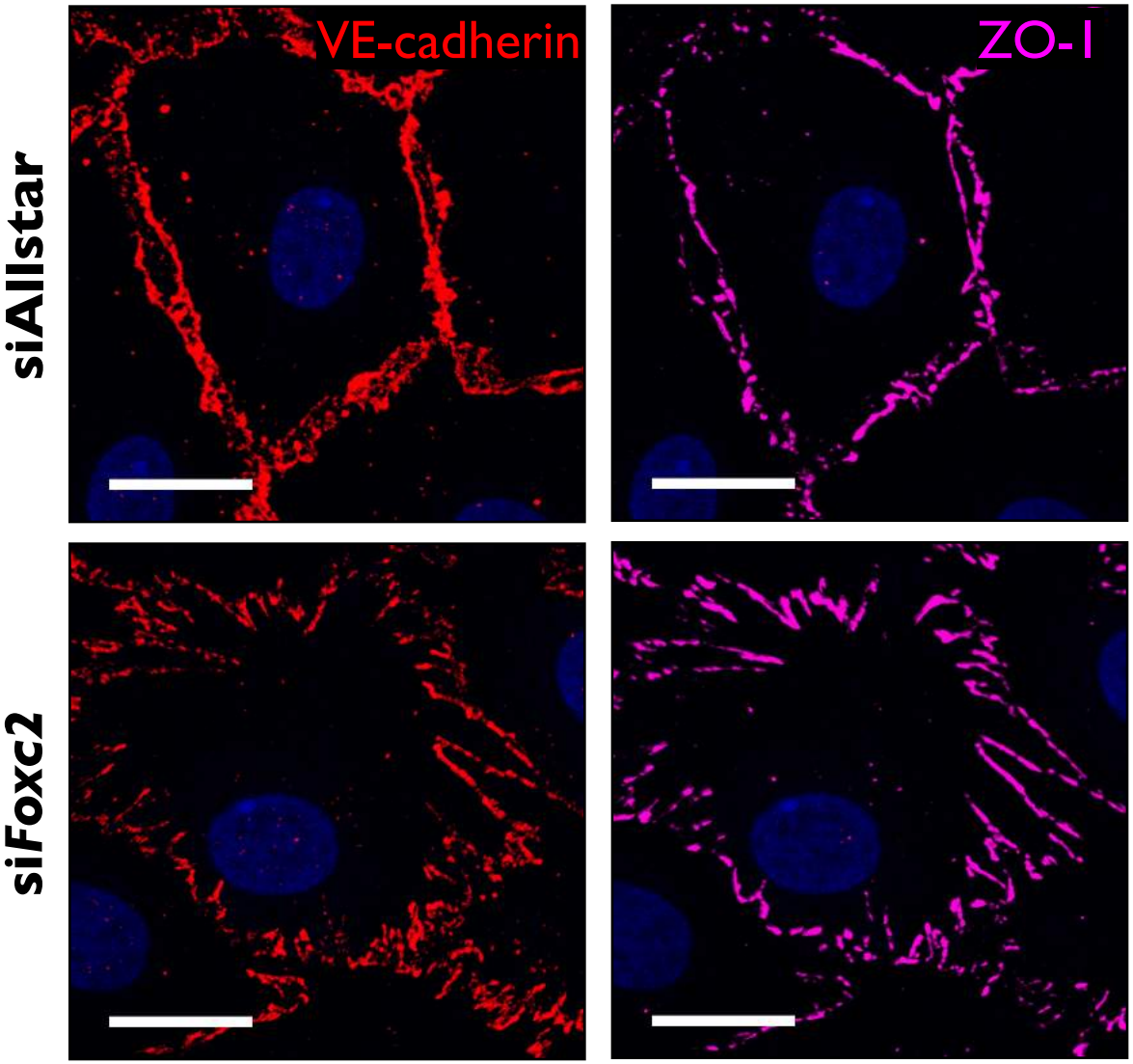
Shear stress → Foxc2 → NF

Can we exploit this fundamental knowledge to propose treatment approaches?

Formation and manitenance of collecting lymphatic vessels and valves

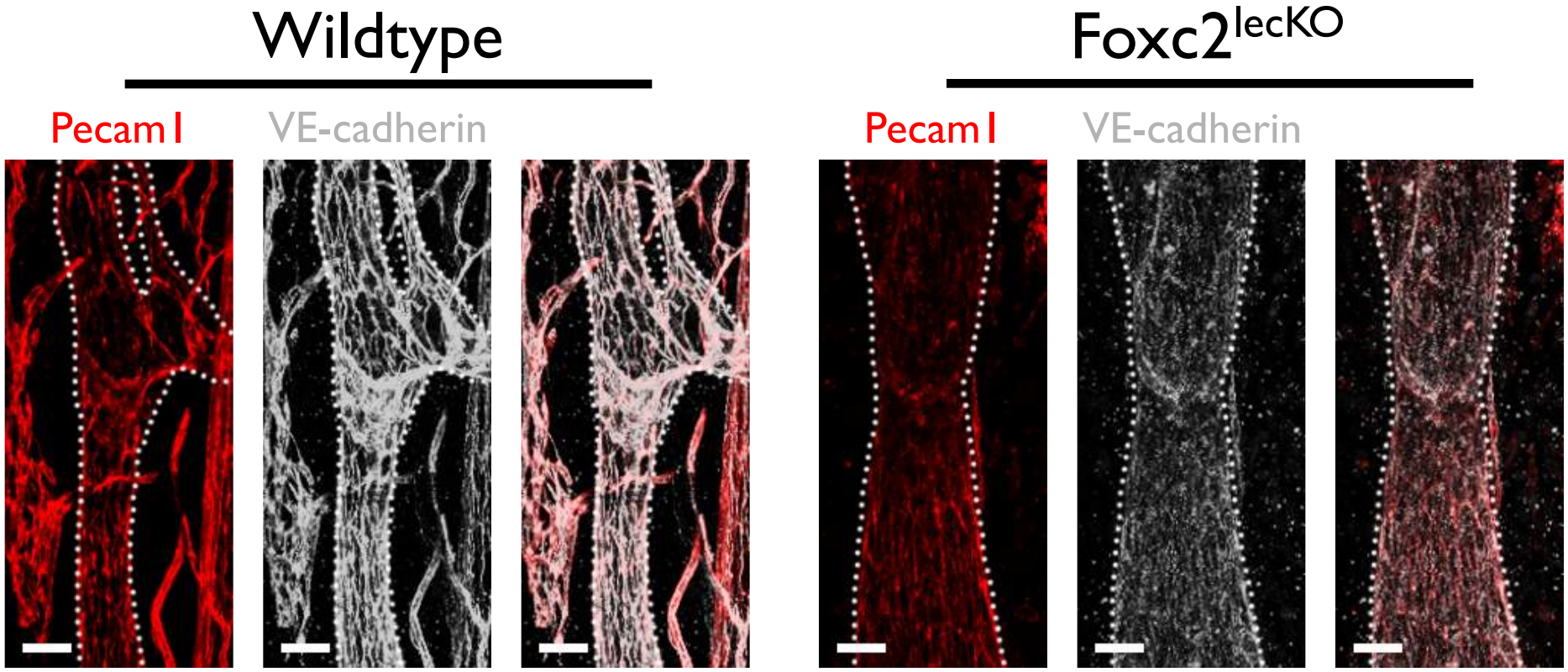


Foxc2 inactivation disrupts cell-cell junctions *in vitro*

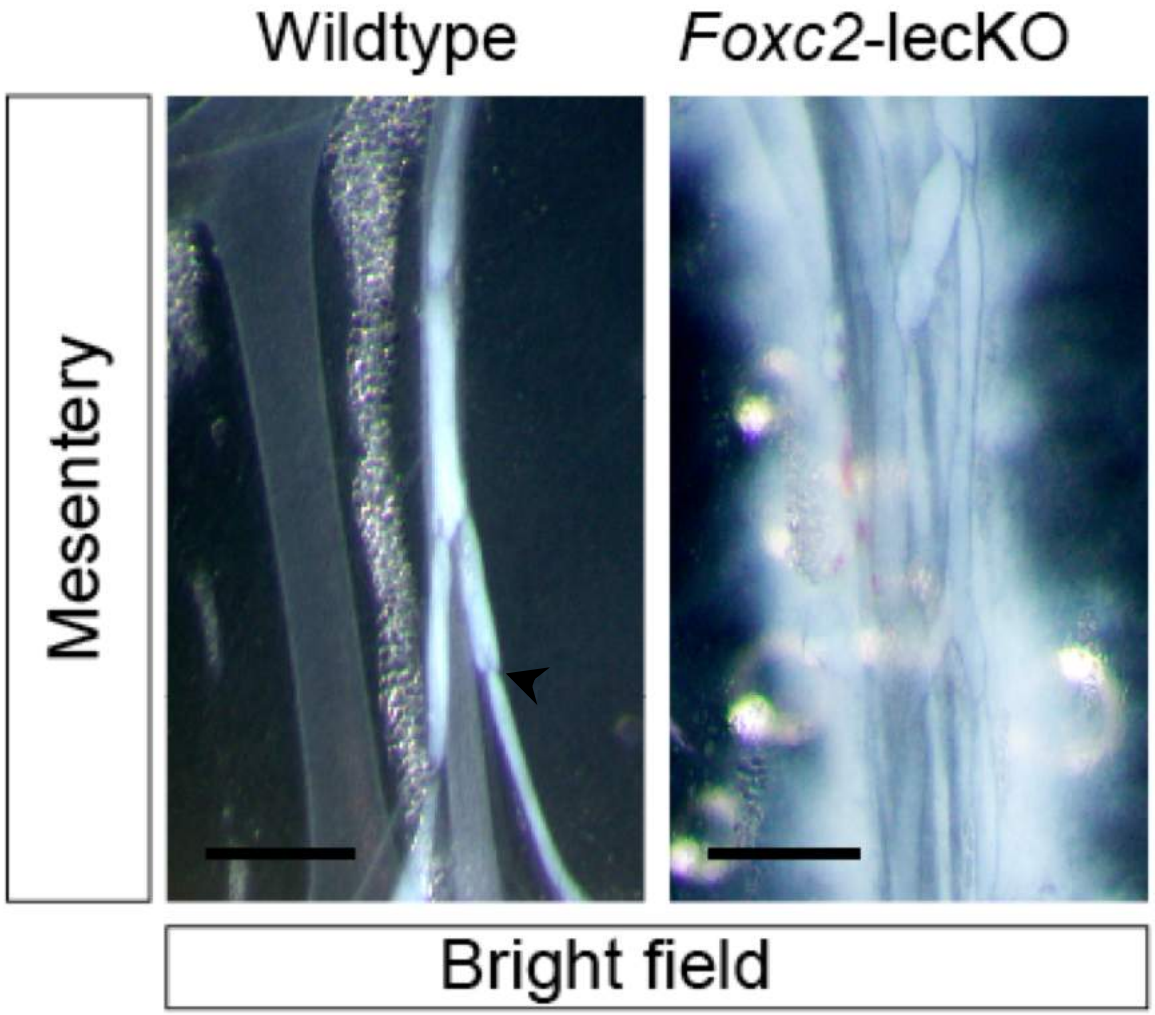


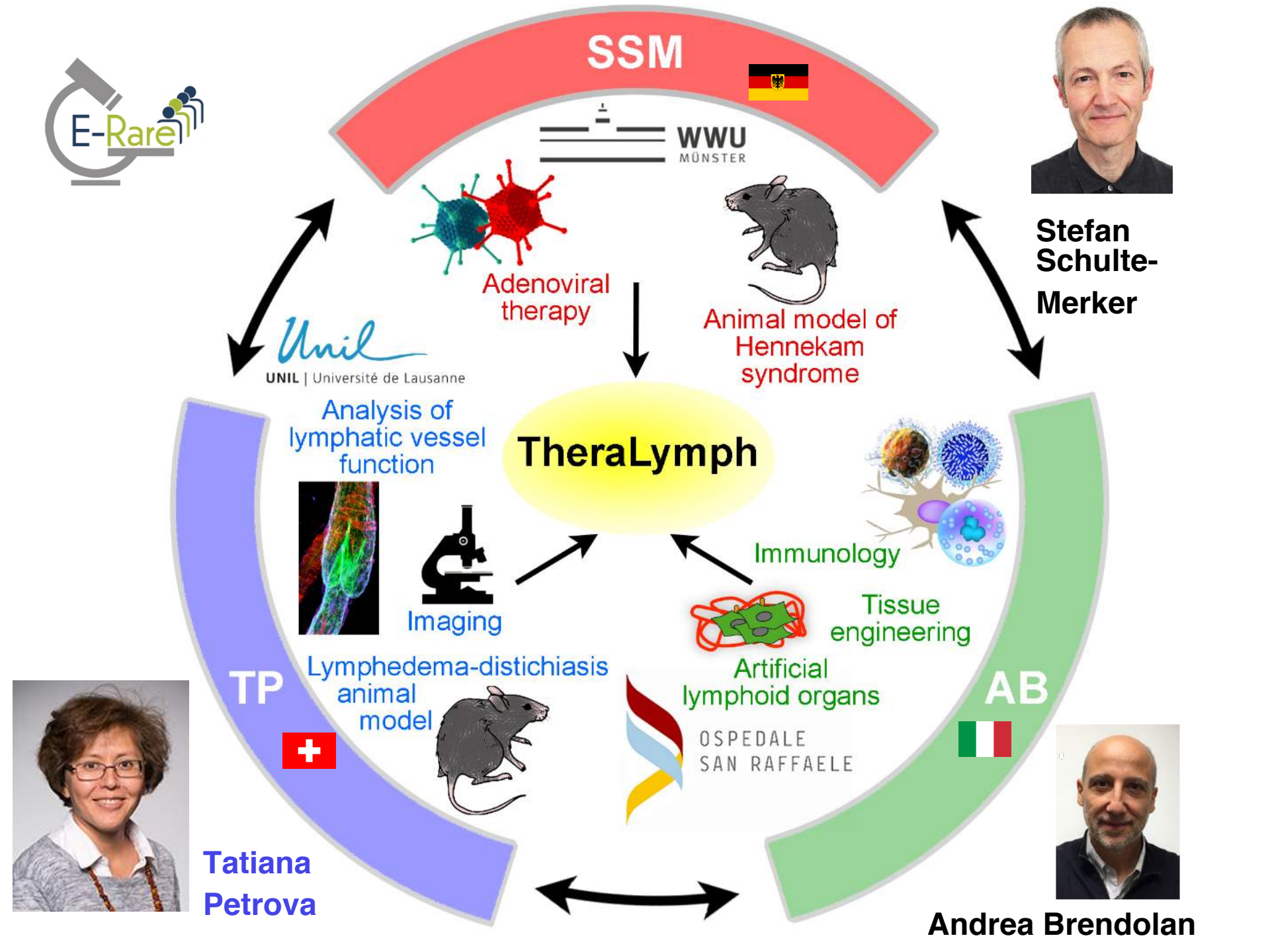
Oscillatory shear stress *(48h - 4 dyn/cm^2 - 4sec)*

Foxc2 inactivation disrupts cell-cell junctions in vivo



Targeting vascular leakage in the animal LD model?




SSM
WWU
MÜNSTER
Adenoviral therapy
Animal model of Hennekam syndrome
Stefan Schulte-Merker
UNIL | Université de Lausanne
Analysis of lymphatic vessel function
TheraLymph
Imaging
Immunology
Tissue engineering
Artificial lymphoid organs
OSPEDALE SAN RAFFAELE
Lymphedema-distichiasis animal model
TP
AB
Tatiana Petrova
Andrea Brendolan

Pharmacological therapy for treatment of lymphedema-distichiasis

What is the potential of repurposing approved drugs to treat lymphedema?

Therapeutic approach	In vitro	In vivo
ROCK inhibitors	✔	X
VE-PTP inhibition	X	
Macrophage recruitment		X
VEGFA driven vascular permeability		X
VEGFC/VEGFR-3 blockade		✔

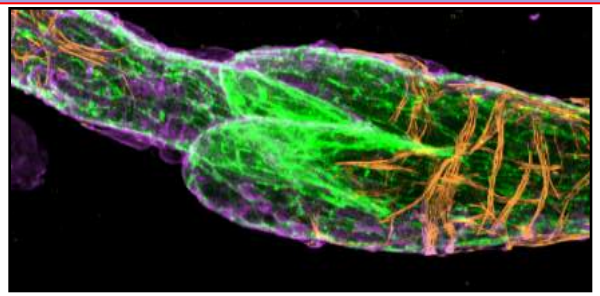
Cdk5

Shear stress

Foxc2 → **Cell-cell junctions**

Calcineurin/NFAT ⇄ Connexin37

Formation and maintenance of collecting lymphatic vessels and valves



Pathological states:
Lymphedema, chylous effusions and inflammation

Lab:
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Amelie Sabine
Cansaran Saygili

Jeremiah Bernier-Latmani
Christophe Cisarovsky
Suzel Davanture
Alejandra Gonzalez
Stefanie Hendrikx
Borja Luri-Prat
Simone Ragusa
Celine Beauverd
Laureline Wetterwald

Past members
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Camilla Norrmèn

FOXC2

Collaborators:
Brenda Kwak
Friedemann Kiefer

Kari Alitalo
Terhi Kärpanen
Lydia Sorokin

Marcus Fruttiger
Naoyuki Miura
Ralf Adams
Taija Mäkinen

Mouse model

Sponsors: Swiss National Science Foundation, NCCR Molecular Oncology, Swiss Cancer League, AICR Medic Foundation, Lymphatic Research Foundation, Emma Muschamp Foundation, Leenaards Foundation, Gebert Rüf Foundation