

Elsevier Editorial System(tm) for Biochemical and Biophysical Research
Communications
Manuscript Draft

Manuscript Number:

Title: TRANSCRIPTION FACTOR COUP-TFII IS INDISPENSABLE FOR VENOUS AND LYMPHATIC
DEVELOPMENT IN ZEBRAFISH AND XENOPUS LAEVIS

Article Type: Regular Article

Keywords: COUP-TFII, endothelial specification, (lymph)angiogenesis, zebrafish, Xenopus laevis, Prox1

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W.J. Lennarz

Editor-in-Chief, *Biochemical and Biophysical Research Communications*

May 18, 2011

Dear Dr. Lennarz,

Please, find enclosed our manuscript entitled 'TRANSCRIPTION FACTOR COUP-TFII IS INDISPENSABLE FOR VENOUS AND LYMPHATIC DEVELOPMENT IN ZEBRAFISH AND *XENOPUS LAEVIS*' by Aranguren *et al.*, to be considered for review at *Biochemical and Biophysical Research Communications*. We think that our paper is of great significance to the readers of *the Journal* since it reports unprecedented evidence for a coordinating role for the transcription factor COUP-TFII (also known as NR2F2) in arterio-venous and veno-lymphatic endothelial cell decisions during development in two small animal models, *Danio rerio* (zebrafish) and *Xenopus laevis* (frogs). To elucidate the role of NR2F2 in these models, we used a morpholino knockdown approach and applied newly developed protocols to specifically sort (lymphatic) endothelial cells from zebrafish embryos and *Xenopus* tadpoles. We chose to combine these models since the zebrafish has been instrumental to study the involvement of genes of interest in arterio-venous specification, while the *Xenopus* model allows for a more in-depth mechanistic/functional analysis of lymphatic development. Our studies demonstrate that, like in mice, NR2F2 is indispensable for proper arterio-venous and veno-lymphatic fate decisions and hence its absence results in severe (cardio)vascular and lymphatic defects. Furthermore, the studies in *Xenopus* NR2F2 morphants allowed us to demonstrate a role for this transcription factor in lymphatic endothelial cell migration *in vivo*, which was previously only demonstrated *in vitro*. Therefore, we further underscore the importance of small animal models in elucidating mechanisms of (lymph) vascular development and have shown that the role of NR2F2 in (lymph)vascular development is evolutionary conserved across vertebrates.

In case the manuscript is subject to external review, we would like to exclude the following person as a reviewer due to conflict of interest: Dr. Brant Weinstein, National Institute of Child Health and Human Development, NIH, Bethesda, USA. We hope that you will find our manuscript suitable for review in *Biochemical and Biophysical Research Communications*. None of the authors has competing financial interests, and all authors concur with submission of the paper to *the Journal*. The data herein have not been previously reported nor are they under consideration for publication elsewhere.

Hoping for your positive reply and thanking you for your consideration,

Yours sincerely,

Aernout Luttun, on behalf of all co-authors

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HIGHLIGHTS

- COUP-TFII deficiency in zebrafish affects arterio-venous EC specification
- COUP-TFII is indispensable for lymphatic development in zebrafish
- COUP-TFII knockdown in *Xenopus* disrupts lymphatic EC differentiation and migration
- COUP-TFII's role in EC fate decisions is evolutionary conserved

**TRANSCRIPTION FACTOR *COUP-TFII* IS INDISPENSABLE FOR VENOUS AND LYMPHATIC
DEVELOPMENT IN ZEBRAFISH AND *XENOPUS LAEVIS***

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ABSTRACT

Transcription factors play a central role in cell fate determination. Gene targeting in mice revealed that Chicken Ovalbumin Upstream Promoter-Transcription Factor II (COUP-TFII¹, also known as Nuclear Receptor 2F2 or NR2F2) induces a venous phenotype in endothelial cells (ECs). More recently, NR2F2 was shown to be required for initiating the expression of *Prox1*, responsible for lymphatic commitment of venous ECs. Small animal models like zebrafish embryos and *Xenopus laevis* tadpoles have been very useful to elucidate mechanisms of (lymph)vascular development. Therefore, the role of NR2F2 in (lymph)vascular development was studied by eliminating its expression in these models. Like in mice, absence of NR2F2 in zebrafish resulted in distinct vascular defects including loss of venous marker expression, major trunk vessel fusion and vascular leakage. Both in zebrafish and *Xenopus* the development of the main lymphatic structures was severely hampered. NR2F2 knockdown significantly decreased *prox1* expression in zebrafish ECs and the same manipulation affected lymphatic (L)EC commitment, migration and function in *Xenopus* tadpoles. Therefore, the role of NR2F2 in EC fate determination is evolutionary conserved.

KEYWORDS: COUP-TFII, endothelial specification, (lymph)angiogenesis, zebrafish, *Xenopus laevis*, *Prox1*

¹**NON-STANDARD ABBREVIATIONS:** Chicken Ovalbumin Upstream Promoter-Transcription Factor II (COUP-TFII); Nuclear Receptor 2F2 (NR2F2); endothelial cells (ECs); venous ECs (vECs); lymphatic ECs (LECs); forkhead box (Fox); SRY-related HMG box F (SoxF); embryonic day (E); whole-mount in situ hybridization (WISH); hours/days-post-fertilization (h/dpf); tetramethyl-rhodamine-isothiocyanate (TRITC); blood vascular ECs (BECs); parachordal lymphangioblast (PL); thoracic duct (TD); dorsal aorta (DA); posterior cardinal vein (PCV); ventral caudal lymphatic vessel (VCLV); dorsal caudal lymphatic vessel (DCLV); gridlock (gri); vascular endothelial growth factor (VEGF); VEGF-receptor 3 (VEGFR3); neural tube (NT); morpholino (Mo); non-silencing (ns).

INTRODUCTION

NR2F2 belongs to a family of orphan receptors that play key roles in neuronal development, organogenesis, cell fate determination and metabolic homeostasis [1,2]. Targeted deletion in mice revealed its absolute requirement for blood vascular and cardiac development [3]. The expression of NR2F2 in the blood vascular endothelium is restricted to veins and its specific deletion in ECs demonstrated a cell-autonomous role for this transcription factor in the induction of venous identity through inhibition of Notch activity in venous (v)ECs, thereby blocking the arterial signaling cascade [4]. Other transcription factors have been associated with arterio-venous specification, including forkhead box (Fox) and SRY-related HMG box F (SoxF) proteins (i.e. Sox7, -17 and 18) [5,6].

LECs arise by trans-differentiation from vECs [7]. Again, transcription factors coordinate this cell fate transition. At embryonic day (E)9 in mice, Sox18 expression becomes apparent in a subset of cells in the anterior cardinal vein in which it induces the lymphatic 'master-switch' Prox1 around E9.75, after which these cells migrate outwards to form lymph sacs and acquire expression of LEC markers [7,8]. NR2F2, being highly expressed in all vECs around that time, is responsible, perhaps in synergy with Sox18, for the induction and temporary maintenance of Prox1 expression [9]. NR2F2 and Prox1 interact at the protein level and together co-determine LEC marker gene expression [10,11].

The zebrafish embryo has been instrumental to document the role of many factors mentioned above in arterio-venous or veno-lymphatic specification. Gain- and loss of function studies have established the arterial sonic hedgehog-vascular endothelial growth factor (VEGF)-Notch induction cascade (reviewed in [6]) and the involvement of Sox7/18 in arterio-venous specification [5]. We recently introduced *Xenopus laevis* as an elegant model to unravel mechanisms of lymphatic development [12]. Morpholino knockdown in both small animal models documented

the conserved role of Prox1 in lymphatic development [12,13,14]. Intriguingly, despite its coordinating role in arterio-veno-lymphatic specification evident from mouse studies, knockdown studies in small animal models in which the involvement of NR2F2 in EC specification was evaluated have not been reported. Zebrafish and *Xenopus* NR2F2 orthologues, seven-up(40) and xCOUP-TFB, respectively, are nearly 100 % identical in their DNA and ligand binding domains to their mammalian counterpart, suggesting a well-conserved role across vertebrates [1]. Here, we eliminated NR2F2 in zebrafish and *Xenopus* embryos by morpholino knockdown and report its effects on (lymph)vascular development in these models.

MATERIALS AND METHODS

Whole-mount in situ hybridization

Wild-type AB zebrafish embryos were fixed overnight and hybridized with antisense probes for *nr2f2*, *ephrinb2a*, *flt4*, *ephb4a* and *gridlock*, as described [15]. Imaging was performed using a Zeiss Lumar V.12 stereomicroscope (Carl Zeiss). For detailed analysis, stained embryos were paraffin-embedded, sectioned and counterstained with nuclear fast red. WISH for *prox1* and *nr2f2* in *Xenopus* tadpoles was performed, as described [12]. Probes were made in-house and reference sequences are provided in the Supplement. Analysis of LEC commitment and migration on tadpoles processed for *prox1* WISH was performed as described in the Supplement.

Fluorescence activated cell sorting of zebrafish and *Xenopus* ECs

Single cell suspensions of 20 or 48 hours-post-fertilization (hpf) *Tg(Fli1:eGFP)^{y1}* or wild-type AB zebrafish embryos were obtained by trypsinization. Vascular (eGFP⁺) and non-vascular (eGFP⁻) cell fractions were sorted with high purity (Fig.S4) using a FACS Aria II device (Beckton Dickinson). To specifically label lymph vessels, *Xenopus* tadpoles at stage (ST)42-44 (staging was done according to Nieuwkoop and Faber, as described [12]) were injected in the pericardial sac with tetramethyl-rhodamine-isothiocyanate-dextran (TRITC-dextran, 2000 kDa; Fig.S5A) and 1 day later TRITC⁺eGFP⁺ LECs and TRITC⁻eGFP⁺ blood vascular ECs (BECs; Fig.S5B) were sorted from monocellular suspensions generated by trypsinization. qRT-PCR on sorted fractions was performed to estimate enrichment for (L)EC markers (Fig.S5C and Fig.S1E).

Morpholino knockdown

Zebrafish lines used were *Tg(Fli1:eGFP)^{y1}* and *Tg(Gata1:dsRed;Fli:eGFP)*. Embryos were kept at 28°C in 0.3x Danieau/0.003% 1-phenyl-2-thio-urea to prevent pigmentation. For *Xenopus* studies, a transgenic *Tg(Flk1:eGFP)* *Xenopus laevis* line

expressing eGFP in blood/lymphatic vasculature was used [16]. Eggs were kept in 0.1x MMR at 18°C until gastrulation was completed and from there on at 22°C, as described [12]. Morpholinos targeting either the translational initiation site or the first exon-intron boundary of *nr2f2* (GenBank ID: NM_131183.1 for zebrafish, NM_001087019.1 for *Xenopus laevis*) and standard control morpholino were purchased from Gene Tools (LLC, Corvallis). Sequences are listed in the Supplement. Morpholinos were injected into single- to two-cell stage zebrafish embryos or in two-cell stage frog embryos [12,15]. Animal procedures were performed according to the guidelines of the Institutional Animal Care and Use Committee of KULeuven.

Phenotypic scoring

Live screening or confocal imaging of *Tg(Fli1:eGFP)^{y1}* zebrafish embryos or *Tg(Flk1:eGFP)* *Xenopus* tadpoles was performed using a Zeiss Lumar V.12 fluorescence stereomicroscope equipped with a Zeiss AxioCam MrC5 digital camera or a laser-scanning microscope LSM510, respectively. In some cases, *Tg(Gata1:dsRed;Fli:eGFP)* zebrafish were used to document extravasation of DsRed blood cells out of eGFP⁺ blood vessels. For screening of parachordal lymphangioblast string (PL; at 52 hpf) or thoracic duct (TD) formation (at 6 dpf) in *Tg(Fli1:eGFP)^{y1}* embryos, the percentage of PL or TD formation was quantified in 10 consecutive somite segments. For phenotyping of *Xenopus Tg(Flk1:eGFP)* tadpoles, the eGFP⁺ main lymphatic vessels in the trunk region were scored as described in the Supplement (Fig.S3). Functionality of these vessels was evaluated by lymphangiography on anesthetized tadpoles at ST46 [12].

RNA isolation and qRT-PCR

Total RNA from sorted cell lysates was extracted using RLT lysis buffer (Qiagen). mRNA was reverse transcribed using Superscript III Reverse Transcriptase (Invitrogen) and cDNA underwent 40 rounds of amplification on an ABI PRISM 7700

cycler (Perkin Elmer/Applied Biosystems). Primers used for amplification are listed in the Supplement. mRNA levels were normalized using *ef1 α* as housekeeping gene.

Statistical analysis

Data, expressed as mean \pm SEM comparing two groups were analyzed by Student's *t*-test. For phenotypic studies, the penetrance of the phenotype, the number of embryos/tadpoles exhibiting the different severities of morphant phenotype were counted and Chi-square analysis was used to determine whether this fraction differed between control or dose groups. SPSS software was used for statistical analyses and differences were considered significant when $P < 0.05$.

RESULTS

Nr2f2* is expressed in vECs and LECs in zebrafish and *Xenopus

While the expression of seven-up(40) (which we will term zebrafish '(z)NR2F2') and xCOUP-TFB (which we will refer to as *Xenopus* '(x)NR2F2') has been well described for the central nervous system [1,17], their expression pattern in the vascular tree has not been documented. By WISH at 48 hpf we found that, like in mouse embryos [4], expression of *znr2f2* in the major trunk vessels (the dorsal aorta (DA) and the posterior cardinal vein (PCV)) was restricted to ECs in the PCV (Supplementary Fig.S1A,B; all supplementary items are designated 'S'). In agreement with its expression in spinal cord motor neurons in mice [1], we also found expression in the neural tube (Fig.S1A,B).

Lymphatic development in zebrafish begins around 30 hpf with the formation of a transient bilateral structure of LEC precursors at the horizontal myoseptum, the 'parachordal lymphangioblast (PL) string' [15,18]. *Znr2f2* was expressed there at 48 hpf (Fig.S1B). Beyond 60 hpf, these LEC precursors migrate dorsally and ventrally, the latter giving rise to the thoracic duct (TD), just underneath the DA. At 6 dpf *znr2f2* was also detected in the TD (Fig.S1C). Like in zebrafish and mice, *xnr2f2* expression was restricted to venous (i.c. the PCV) and lymphatic structures (i.c. the ventral caudal lymphatic vessel or VCLV), but absent from the DA (Fig.S1D). *Xenopus* LECs (sorted after specifically labelling them with a fluorescent compound) expressed *xnr2f2* to a comparable extent as BECs (Fig.S1E).

zNR2F2 knockdown causes cardiovascular defects

Similar to mice [3], zNR2F2 silencing, induced by injecting a morpholino against the ATG region of *znr2f2* (zNR2F2^{ATG}) resulted in a dose-dependent cardiovascular phenotype, including vascular leakage (Fig.1A), pericardial edema, arterio-venous segregation faults, intersomitic vessel defects and abnormal anatomy of the caudal vascular labyrinth (Fig.S2). WISH and qRT-PCR analysis on eGFP⁺

ECs sorted from 48 hpf transgenic *Tg(Fli:eGFP)^{y1}* embryos expressing eGFP under control of the EC-specific Fli-promoter revealed that the vascular defects were in part due to a shift in arterio-venous gene expression, i.e. selective downregulation of vEC markers without detectable ectopic expression of arterial markers in veins (Fig.1B-F and not shown). These results were confirmed with a splice-site morpholino (not shown). Even though flow may co-determine arterio-venous identity [19], the altered expression was not secondary to the cardiac phenotype, as it was also apparent at 20 hpf, before the onset of flow (Fig.1B).

zNR2F2 knockdown severely hampers lymphatic development

Given its expression throughout zebrafish lymphatic development, we analyzed whether zNR2F2 knockdown affected the formation of the zebrafish lymphatic system. Consistent with a role for zNR2F2 in LEC specification, expression of the lymphatic master-switch gene *prox1a* was significantly (~70 %) reduced in sorted ECs from zNR2F2 morphants at 48 hpf (Fig.2A). The recently identified co-orthologue *prox1b* [13] was also downregulated upon zNR2F2 knockdown (not shown). Consistent herewith, subsequent formation of the lymphatic PL string and TD was severely impaired upon zNR2F2 knockdown (Fig.2B-H). Indeed, at 52 hpf there was a significant and dose-dependent effect on the presence and abundance of the PL string, the latter being absent or only partially developed in 77 % of the high dose zNR2F2 knockdown embryos (Fig.2B,D,E). Later, at 6 dpf, while the TD was completed in all control embryos, TD formation was completely or partially abolished in 85 % of high dose zNR2F2 morphants (Fig.2B,G,H). This effect on TD development was likely underestimated, since, especially at higher morpholino doses, the GFP signal was flawed by trunk edema, which prevented us from analyzing a significant fraction of embryos.

xNR2F2 knockdown impairs LEC commitment, migration and function

Because the zebrafish model does not allow to distinguish between a role for NR2F2 in LEC commitment versus subsequent migration, and since the dynamics of

Prox1 expression can be easily visualized in *Xenopus* [12,16], we also knocked-down NR2F2 in *Tg(Flk1:eGFP) Xenopus laevis* tadpoles. xNR2F2 morpholino knockdown resulted in severe lymphedema (Fig.3A,B). Analysis of *prox1* by WISH revealed that LEC specification was significantly affected upon xNR2F2 silencing at ST32, as shown by a reduced signal in area 1, defined as the area where LEC commitment occurs ($[\mu\text{m}^2 \times 10^3]$: 6 ± 1 in xNR2F2 morphants vs. 12 ± 1 in control; $n=17-22$; $*P<0.001$; Fig.3C,D). Moreover, dorsal migration of LECs at ST35/36 in the tail region of xNR2F2 morphants was severely dampened (Fig.3E-G). Consequently, lymphatic development was severely impaired in xNR2F2 morphants as shown by the partial absence or disorganized structure of the dorsal caudal lymphatic vessel (DCLV) and VCLV both in the anterior and posterior trunk region (Fig.S3). In addition, in case lymph vessels did develop, their draining capacity was impaired (Fig.3H,I).

DISCUSSION

From studies in mice and human cells, NR2F2 is known to take up a central position in EC fate decisions [4,7,10,11]. Here, we demonstrated that it has a comparable role in *Danio rerio* (zebrafish), a species that emerged on earth millions of years earlier. Nevertheless, careful analysis of the underlying mechanisms revealed that subtle differences have emerged during evolution. Eliminating NR2F2 expression resulted in a shift towards the arterial fate in both mice and zebrafish, however, this shift was accomplished in a different way. While NR2F2 deficiency in mouse ECs causes ectopic arterial expression in veins [4], we observed downregulation of venous genes without detectable ectopic expression of arterial markers in zebrafish. In addition, while in mice, a working model was presented in which NR2F2 induces a vEC phenotype by blocking the Notch pathway [4], in zebrafish we did not find support for such a scenario since the expression of *gridlock* (*grl*), the zebrafish orthologue of *Hey2* (a downstream target of the Notch pathway), was unaltered upon zNR2F2 knockdown. This apparent contradiction can be resolved by the observation that in zebrafish *grl* may not be a downstream target of Notch signaling [20].

In addition to its conserved role in arterio-venous decision-making, the absence of NR2F2 during early zebrafish life severely hampered lymphatic development. Unlike in previous studies [15], where it was possible to titrate the morpholino dose to reveal lymphatic defects in the absence of severe blood vascular abnormalities, we could not find such a dose for the zNR2F2 morpholinos. Hence, we cannot exclude that the absence of lymphatic structures in zNR2F2 morphants was secondary to the defective development of the PCV from which the lymphatic system arises. In *Xenopus* however, blood vessel development was only mildly affected with the morpholino doses we used, suggesting that the severe lymphatic phenotype we obtained following xNR2F2 knockdown was caused by a direct effect on LECs. In agreement, we (unpublished results) and others have shown in human

LECs and transgenic mice that NR2F2 has a cell-autonomous role in these cells [9,21].

Our *Xenopus* studies enabled us to dissect in more detail the mechanisms by which NR2F2 determined lymphatic development. Like in mice, xNR2F2 was indispensable for LEC commitment, which required the induction of prox1 expression in a subset of ECs in the PCV. Once committed to their new fate, LECs move out from the cardinal vein to assemble the first lymphatic structures, which are the lymphatic sacs in mammals [7]. Here, we show that xNR2F2 is also involved in driving LEC migration resulting in the formation of two large lymphatic vessels in the trunk, one in dorsal (DCLV) another in ventral position (VCLV) relative to the PCV. The distance that has to be bridged to reach the dorsal position is however much longer than the ventral position. Hence, the lymphatic defect was in general more severe in the DCLV as compared to the VCLV (absence of the DCLV versus abnormal architecture of the VCLV; Fig.S3), in agreement with previous knockdown studies for factors involved in lymphatic development such as synectin [22], VEGFR3 [12,23] and Liprin β -1 [16]. Also, defects along the anterior-posterior axis were more prevalent in the anterior portion of the trunk (Fig.S3), supposedly because the anterior region is more distant from the region where LEC budding is initiated at ST32 (Fig.3) and since additional commitment/budding sites in the anterior region only emerge later in development [12]. In mice, simultaneous expression of Sox18 and NR2F2 in a subset of ECs in the anterior cardinal vein has raised the hypothesis that both factors cooperate to induce Prox1 expression in these cells [9]. While Sox18 has been knocked down in zebrafish (reviewed in [5]) and *Xenopus* [24], a role for this factor in lymphatic development has not been documented in these small animal models.

In conclusion, the role of NR2F2 in arterio-venous and veno-lymphatic fate decisions is evolutionary conserved. Despite some subtle differences, zebrafish and *Xenopus* tadpoles together have once again proven their value as model organisms

to study the role of a chosen factor in (lymph)vascular development. Moreover, the continuous development of additional tools, such as the availability of transgenic lines or specific labelling techniques in which veins, arteries or lymphatic vessels can be distinguished will significantly boost their use in (lymph)vascular research [16,18].

ACKNOWLEDGEMENTS

We thank P. Vandervoort, T. Vervoort, B. Hoekman and T. Koninckx for technical assistance. This work was supported by grants from the European Commission (FP7-StG-IMAGINED 203291; A.L.; STREP-FP6-StrokeMAP; A.L., M.D., P.C.), a Fund for Scientific Research (FWO) post-doctoral fellowship (A.L.), a post-doctoral grant from the Lymphatic Research Foundation (LRF; X.L.A.), two Agentschap voor Innovatie door Wetenschap en Technologie (IWT) pre-doctoral fellowships (M.B., W.V.), a KULeuven Center of Excellence grant (EF/05/013; A.L.) and an FWO research grant (FWO G069209; M.D., P.C.).

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LEGENDS

Figure 1. zNR2F2 knockdown causes vascular defects

A. Brightfield/immunofluorescence merged image (green=vessels; red=blood) of the trunk of a 48 hpf zebrafish (z)NR2F2^{ATG} morpholino (Mo)-injected *Tg(GATA1:DsRed;Fli:eGFP)* embryo revealing leaky (circled area) vessels. **B.** qRT-PCR on the eGFP⁺ endothelial fraction of 48 hpf *Tg(Fli:eGFP)^{y1}* embryos injected with non-silencing ('ns') Mo (white) or with 3.2 ng zNR2F2 Mo (48 hpf: gray; 20 hpf: black) showing downregulation of venous but not arterial markers. Data expressed as mean % ± SEM vs. ns Mo. (*N*=3-6; **P*<0.05, ***P*<0.01 vs. ns Mo). **C-F.** WISH of 48 hpf embryos revealing downregulation of *flt4* and *ephb4a* expression in the posterior cardinal vein (PCV; indicated by arrowheads) of zNR2F2^{ATG} Mo-injected embryos (D,F) as compared to ns Mo-injected embryos (C,E). Scale bars: 25 μm in A and 200 μm in C-F.

Figure 2. zNR2F2 knockdown severely hampers lymphatic development

A. qRT-PCR on the eGFP⁺ ECs of 48hpf *Tg(Fli:eGFP)^{y1}* embryos injected with non-silencing ('ns') Mo (white) or with zNR2F2 Mo (0.8 ng: gray; 3.2 ng: black) showing downregulation of lymphatic marker *prox1a*. Data expressed as mean % ± SEM vs. ns Mo. (*N*=6; **P*<0.05 vs. ns Mo). **B.** Quantitative analysis of the major lymphatic structures reveals a dose-dependent effect of zNR2F2 Mo on the presence/abundance (defined as number of segments in which the structure was present) of the parachordal lymphangioblast (PL) string (upper panel) and the thoracic duct (TD; lower panel). Data are expressed as % of embryos that featured the PL or the TD in 0 (black), 1-9 (gray) or 10 (white) segments (**P*<0.0001 vs. ns Mo for each dose; numbers above the bars correspond to the number of embryos analyzed). **C-H.** Analysis of 10 consecutive intersomitic segments ('S1-10') for the presence of the PL at 52 hpf (C-E) or the TD at 6 dpf (F-H). Confocal images of the trunk are shown of 52 hpf (D,E) or 6 dpf (G,H) *Tg(Fli:eGFP)^{y1}* embryos injected with ns (D,G) or zNR2F2 Mo (E,H) revealing absence (asterisks in E,H) of the PL string

(green structure in C, arrowheads in D) and the TD (green structure in F, arrowheads in G). Dorsal aorta (DA) indicated by dashed white lines in G,H. Embryo head facing left and dorsal side facing up in D,E,G,H. Scale bars: 50 μ m in D,E,G,H.

Figure 3. xNR2F2 knockdown impairs LEC commitment, migration and function

A,B. Stage (ST)45 tadpoles injected with non-silencing ('ns') (A) or *Xenopus* (x)NR2F2 morpholino (Mo; B) showing edema (arrowheads) in the latter. **C,D.** WISH on ST32 tadpoles injected with ns (C) or xNR2F2 Mo (D), revealing less *prox1* signal in area 1 (a1), indicating reduced LEC commitment [12]. **E-G.** WISH for *prox1* on ST35/36 tadpoles treated with ns (E, white in G) or xNR2F2^{ATG} Mo (F, black in G) and corresponding quantification (G), revealing significantly lower *prox1* signal in area 1 (a1; indicating LEC commitment), a2 and a3 (indicating LEC migration) and a significantly reduced maximal migration distance (MMD) in xNR2F2 morphants (G; data represent mean area or distance \pm SEM; $N=20$; * $P<0.05$, ** $P<0.01$ vs. ns Mo). **H,I.** Lymphangiograms (arrows indicate injection spot) of ST46 *Tg(Flk1:eGFP)* tadpoles revealing draining defects in the VCLV of xNR2F2 Mo-injected tadpoles (M). Bottom panels are higher magnifications of the boxed areas. Tadpole head facing left and dorsal side facing up in C-F and H,I. Scale bars: 200 μ m in E,F; 250 μ m in C,D,H,I and 1 mm in A,B.

FIGURE CAPTIONS

MAIN FIGURES

Figure 1. zNR2F2 knockdown causes vascular defects

This figure illustrates that the absence of NR2F2 expression in zebrafish embryos results in specific vascular defects, including vascular leakage and a shift in arterio-venous gene expression.

Figure 2. zNR2F2 knockdown severely hampers lymphatic development

This figure demonstrates that NR2F2 knockdown in zebrafish embryos affects *prox1* expression and abolishes the development of the main lymphatic structures.

Figure 3. xNR2F2 knockdown impairs LEC commitment, migration and function

This figure shows that NR2F2 knockdown in *Xenopus laevis* tadpoles hampers lymphatic endothelial cell (LEC) commitment and migration, leading to functional defects in the lymphatic vessels.

SUPPLEMENTARY FIGURES

Figure S1. *Nr2f2* is expressed in vECs and LECs in zebrafish and *Xenopus*

This figure documents that the expression of *nr2f2* in zebrafish and *Xenopus laevis* is restricted to venous (vECs) and lymphatic endothelial cells (LECs) and absent in arterial vessels.

Figure S2. zNR2F2 knockdown causes cardiovascular defects

This figure documents that the absence of NR2F2 expression in zebrafish embryos results in specific cardiovascular defects, including pericardial edema, arterio-venous fusion, intersomitic vessel faults and vascular labyrinth abnormalities.

Figure S3. Scoring of lymphatic phenotype upon xNR2F2 knockdown

This figure shows representative images of structural lymphatic defects, depicts the method used to evaluate lymphatic structural defects in *Xenopus laevis* morphants and summarizes the quantification of the lymphatic phenotype in the anterior and posterior tail region.

Figure S4. Endothelial cell sorting from zebrafish embryos

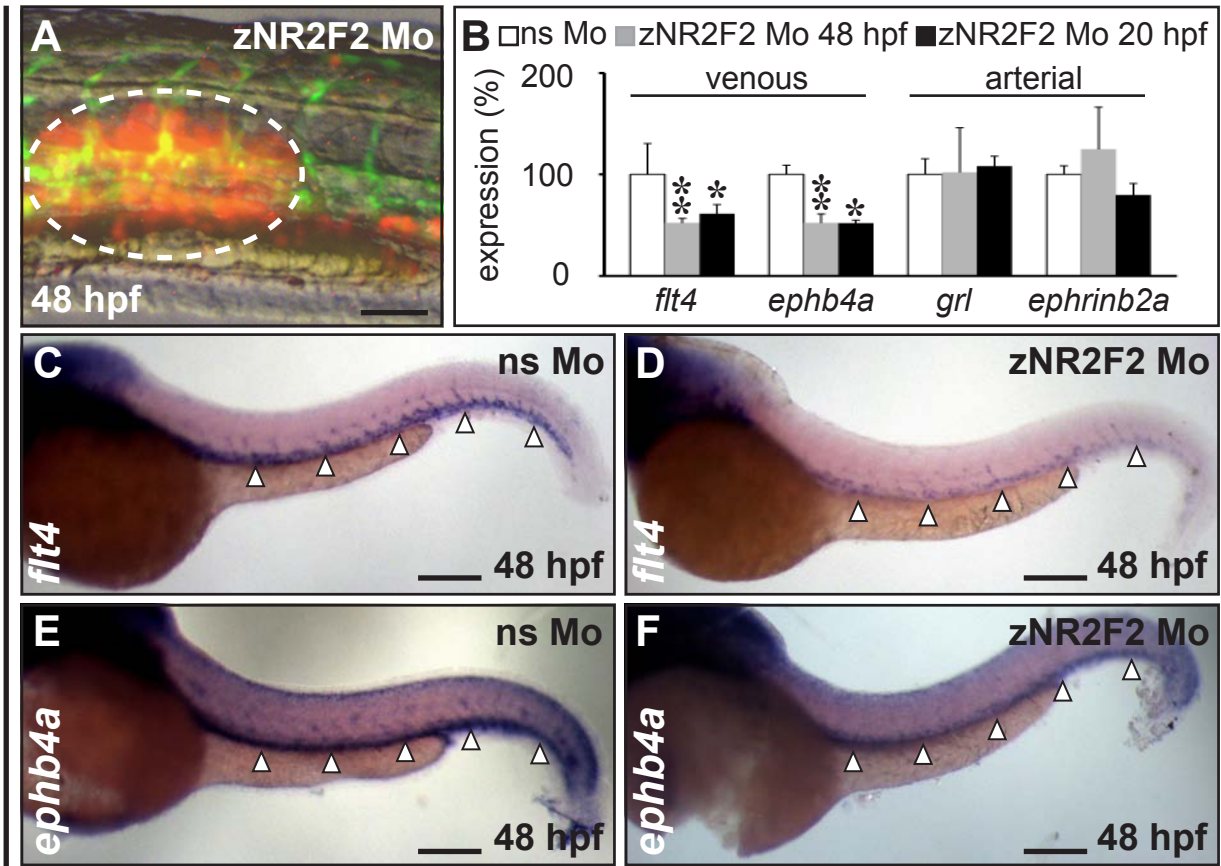
This figure demonstrates the method used to sort endothelial cells from zebrafish embryos.

Figure S5. Endothelial cell sorting from *Xenopus* tadpoles

This figure demonstrates the method used to sort endothelial cells from *Xenopus* tadpoles.

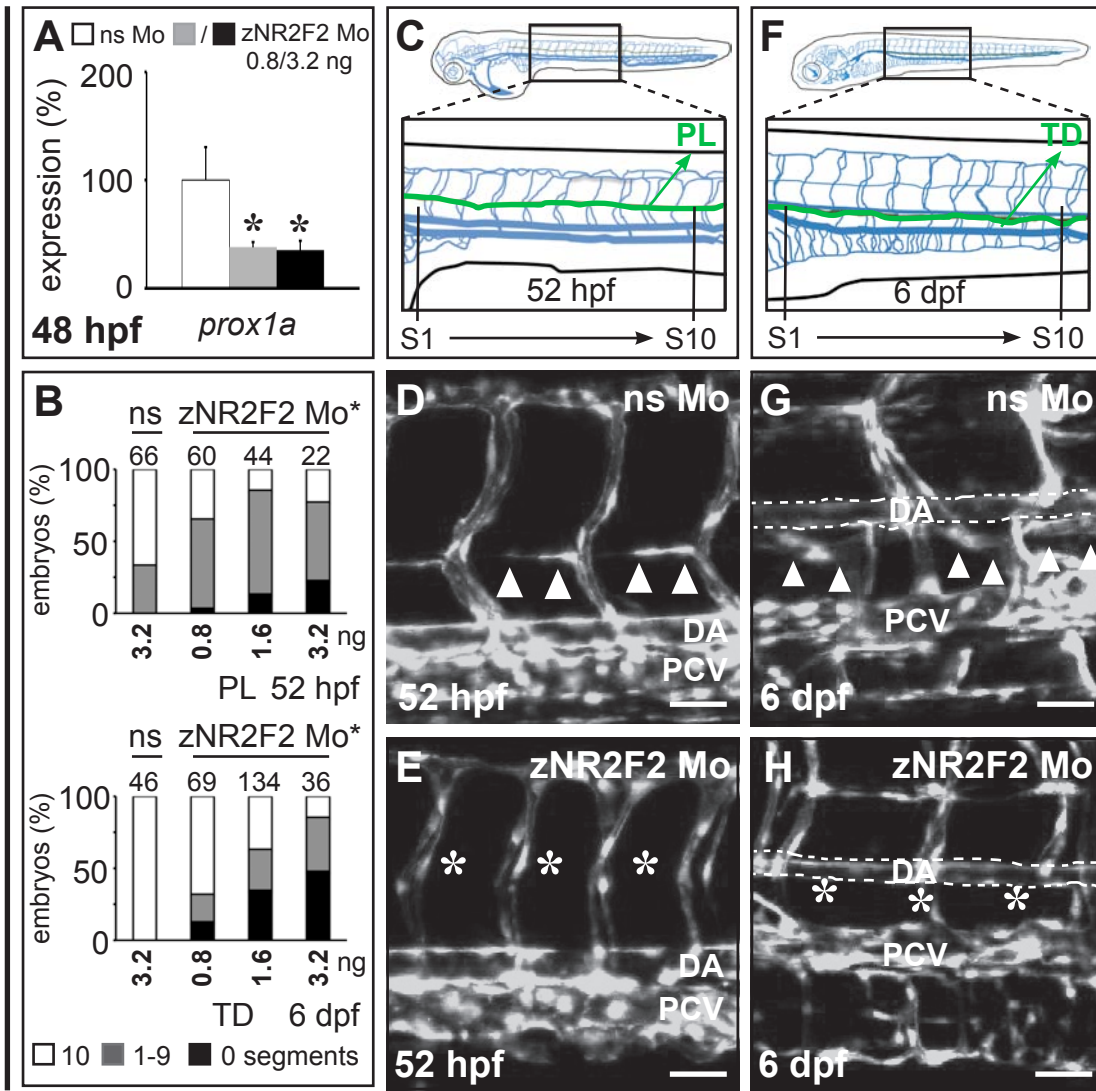
Figure 1

zebrafish (*Danio rerio*)



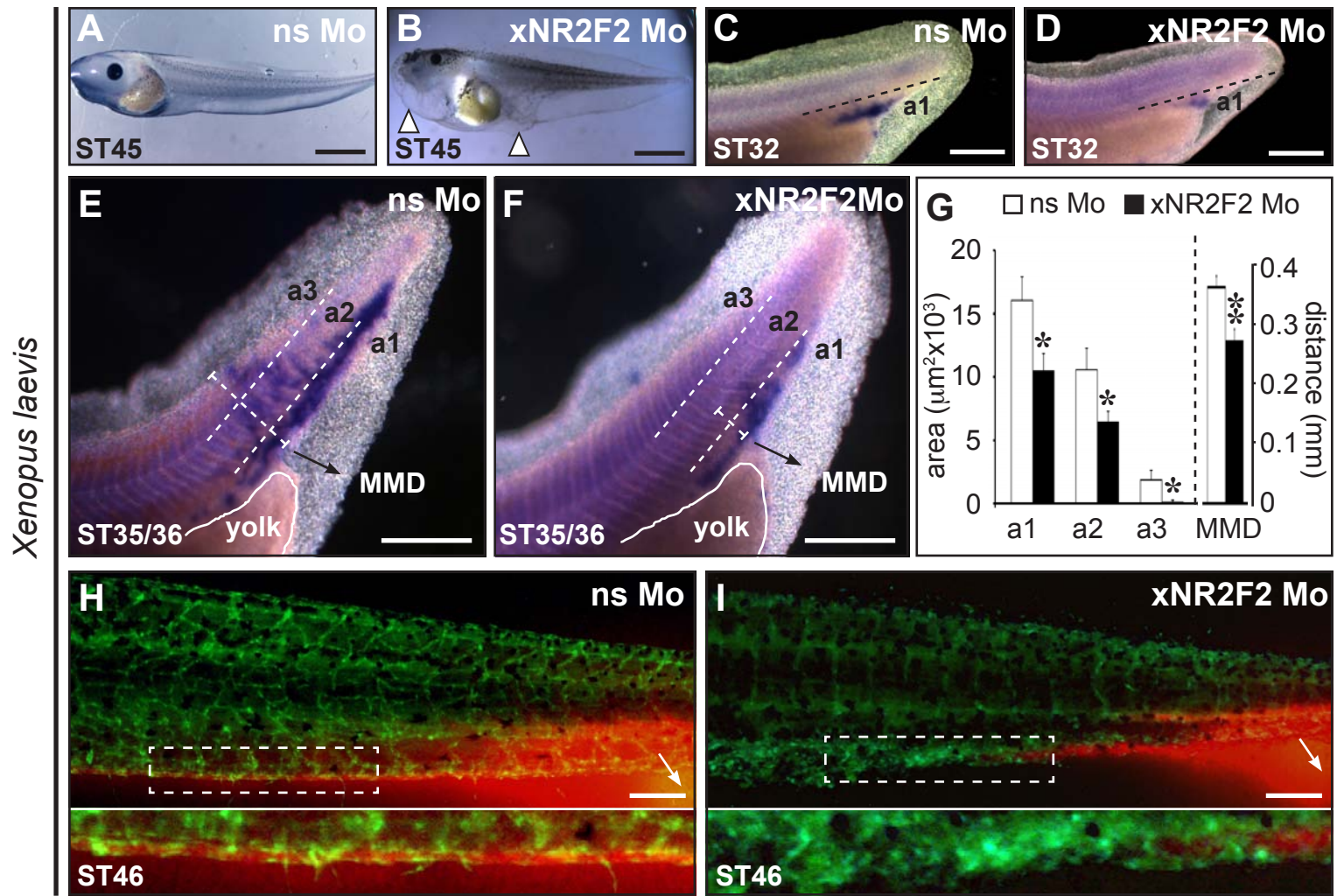
Aranguren et al. Figure 1

zebrafish (*Danio rerio*)



Aranguren *et al.* Figure 2

Figure3



Aranguren *et al.* Figure 3

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