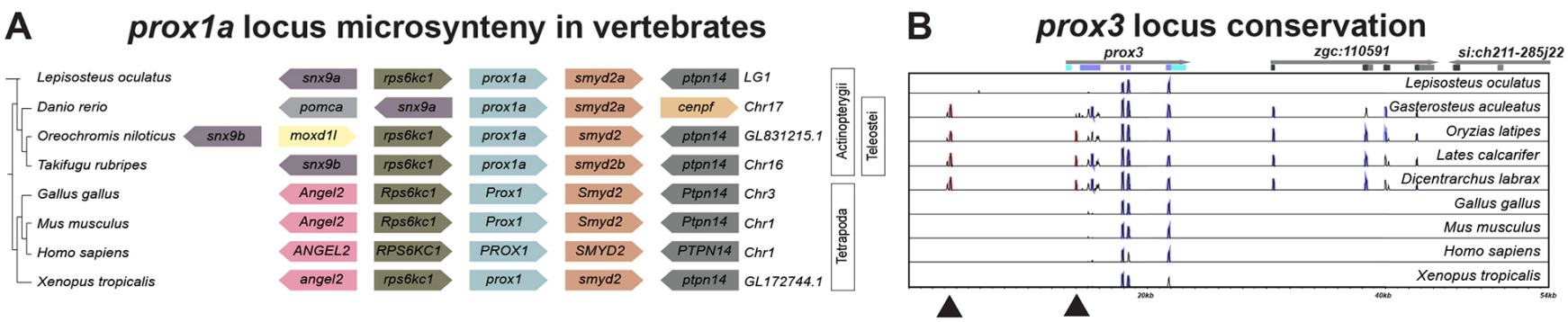

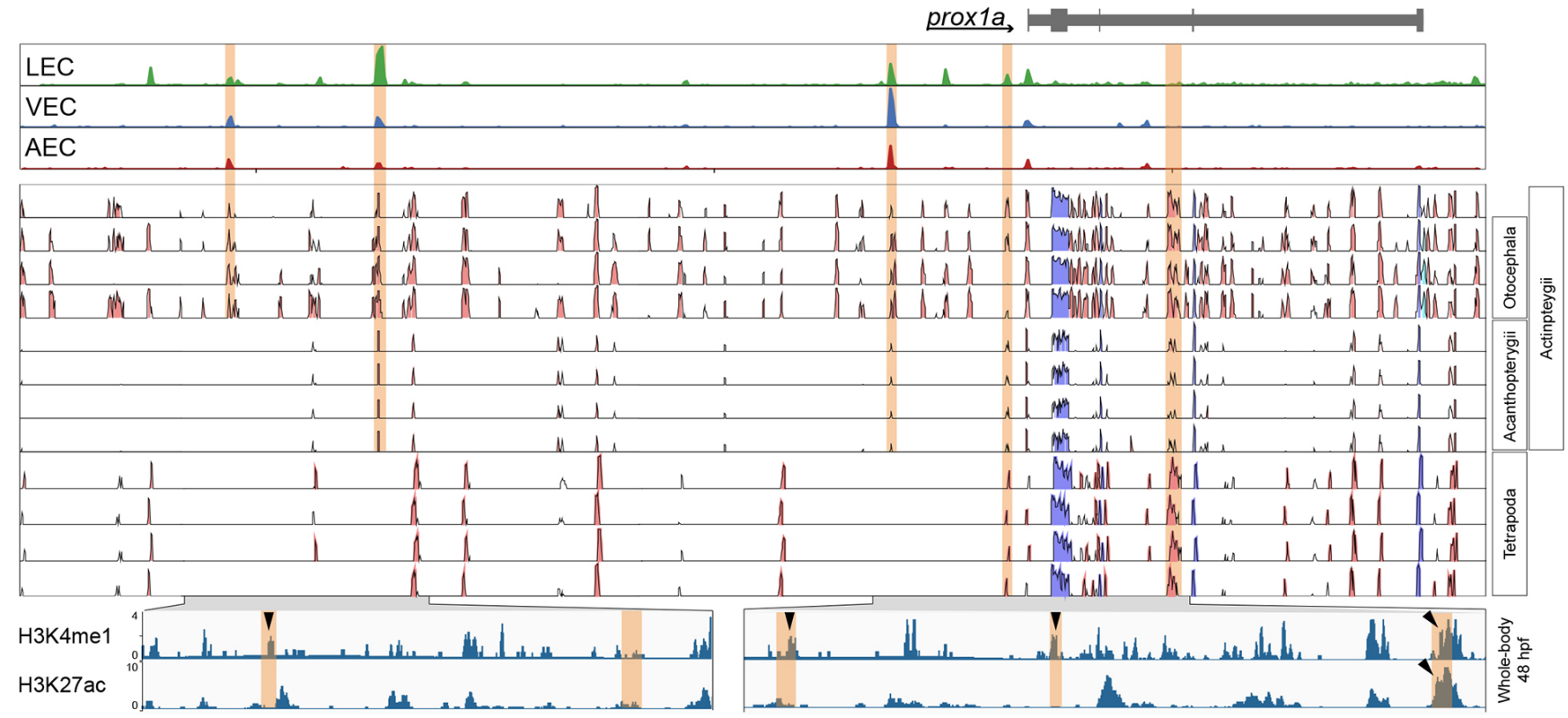

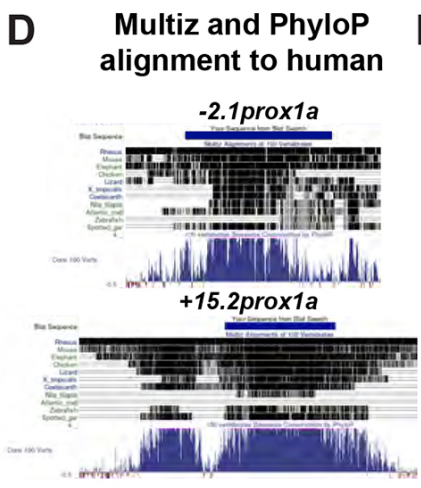

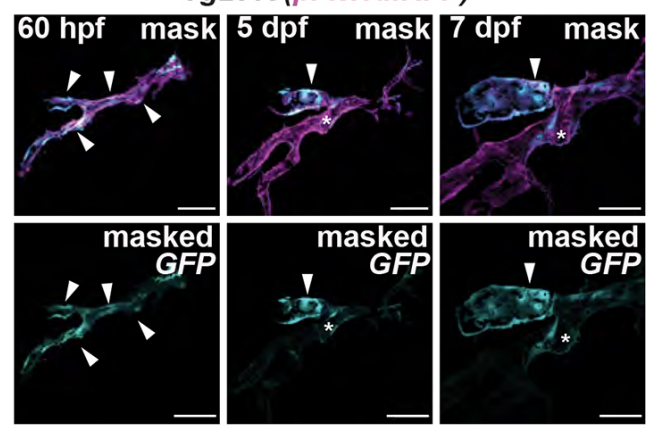

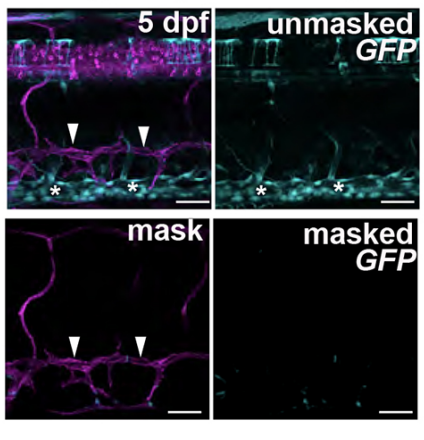

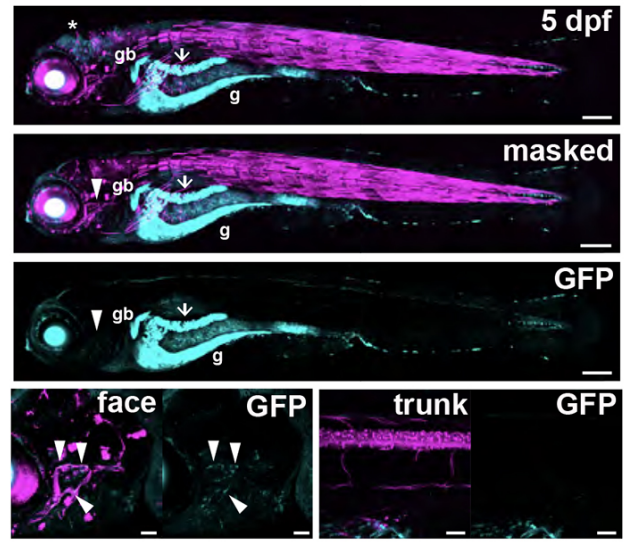

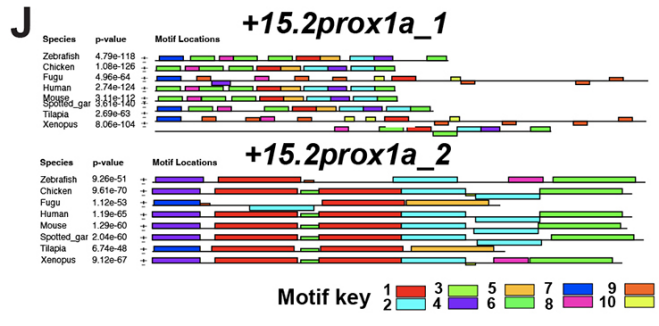

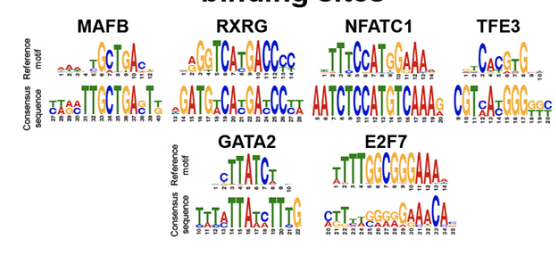

Development • Supplementary information

Fig. S1. CREs identification and additional *+15.2prox1a* enhancer characterisation (A) Microsynteny surrounding the vertebrate *Prox1/prox1a* locus. Downstream of *Prox1/prox1a*, *Smyd2* is found in all analysed sequences. Upstream of *Prox1/prox1a, Rps6ck1* is found in all species, except zebrafish. The annotations used are listed in Table S1.
(B) Conservation analysis of the 54 kbp region surrounding the *prox3* locus comparted to nine vertebrate species. Results are shown compared to zebrafish. Blue peaks: Exons; red peaks: Region of conserved non-coding DNA; black arrowheads: Conserved peaks.
(C) Schematics showing the identified *prox1a* enhancers, the snATAC signature in LEC and VEC, the conservation across Osteichthyes and the H3K4me1 and H3K27ac signature from whole embryos at 48 hpf, indicating primed and active enhancers. Orange: position of the identified enhancers. Arrowheads: H3K4me1 and H3K27ac signals in correspondence to the *prox1a* enhancers.
(D) Multiz alignment and PhyloP tracks from the UCSC Genome browser showing conservation across vertebrates of *+15.2prox1a* and *-2.1prox1a*. Conservation is shown compared to human.
(E) Masked confocal projections of the image in Fig. 1C. Arrowheads: expression in the facial LECs (60 hpf) and FCLV (5 and 7 dpf). Asterisks: Expression in facial lymphatic endothelium.
(F) Confocal projections of VA-L labelled with *Tg(+15.2prox1a:EGFP;XCA:DsRed2)*^{uu7kk} (cyan) and *Tg(prox1a:RFP)*^{nim5} (magenta) at 54 hpf, showing no lymphatic expression.
(G) Confocal projections of the primary head sinus labelled with *Tg(+15.2prox1a_1:EGFP;XCA:DsRed2)*^{uu7kk} at 5 dpf, showing *+15.2prox1a* driven venous expression (arrowhead).
(H) Confocal projections of trunk lymphatics labelled with *Tg(+15.2prox1a:EGFP; XCA:DsRed2)*^{uu7kk} (cyan) and *Tg(prox1a:RFP)*^{nim5} (magenta) at 5 dpf. Arrowheads: lymphatic vessels. Asterisks: posterior cardinal vein.
(I) Embryo labelled with *Tg(+15.2prox1a:basEGFP;ACry:GFP)*^{uu13kk} (cyan) and *Tg(prox1a:RFP)*^{nim5} (magenta). Arrowhead: expression in the FCLV and facial lymphatics. Asterisks: expression in the skin. Arrow: expression in the pancreas. g: expression in the gut. gb: expression in the gallbladder. Scale bar: 200 µm.
(J) Conserved motif structure of the identified lymphatic *+15.2prox1a* enhancer. Motif sequences are listed in Table S4.
(K) Predicted conserved binding site for *+15.2prox1a* in vertebrates. Motif locations are listed in Table S4.
Scales bars represent 50µm unless otherwise stated.

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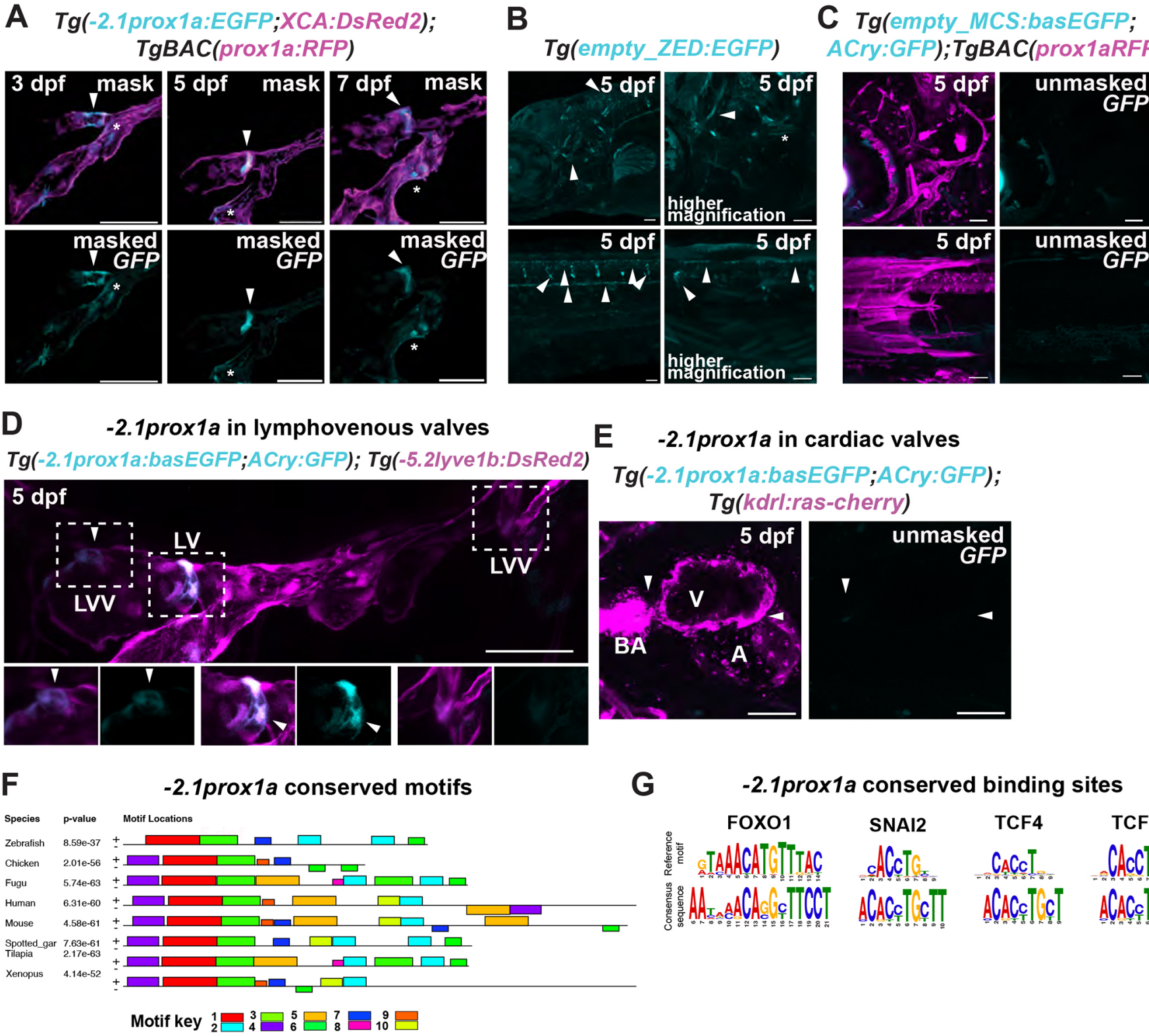


Fig. S2. Additional *-2.1prox1a* enhancer characterisation

(A) Masked confocal projections of the image in Fig. 2A. Arrowheads: Expression in the developing lymphatic valve. Asterisks: Expression in the facial lymphatic endothelium.

(B) Confocal projections of facial (top) and trunk (bottom) lymphatics at 5 dpf labelled with the empty ZED vector as a control. Arrowheads: Neuronal structures.

(C) Confocal projections of facial and trunk lymphatics at 5 dpf labelled with *Tg(prox1a:RFP)*^{nim5} (magenta) and injected with *empty_MSC:basEGFP;ACry:GFP* plasmid to create a transient control.

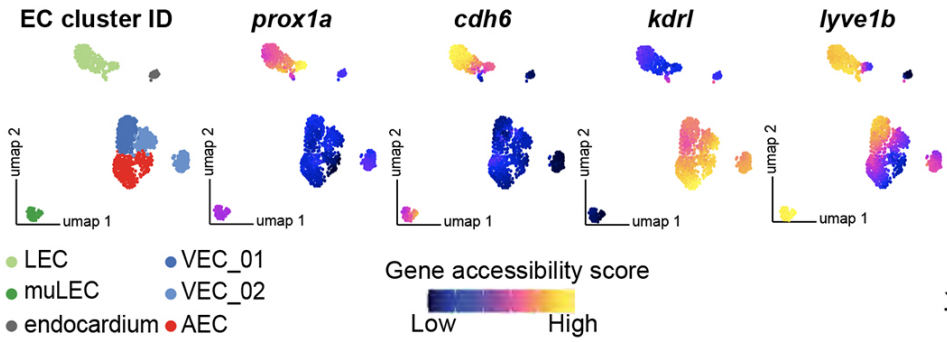
(D) Confocal projections of the LVVs at 5 dpf labelled with *Tg(-2.1prox1a: basEGFP;ACry:GFP)*^{uu10kk} (cyan) and *Tg(-5.2lyve1b:DsRed2)*^{nz101} (magenta). Boxes: LVVs and LV position. Arrowheads: expression in the LVV and LV.

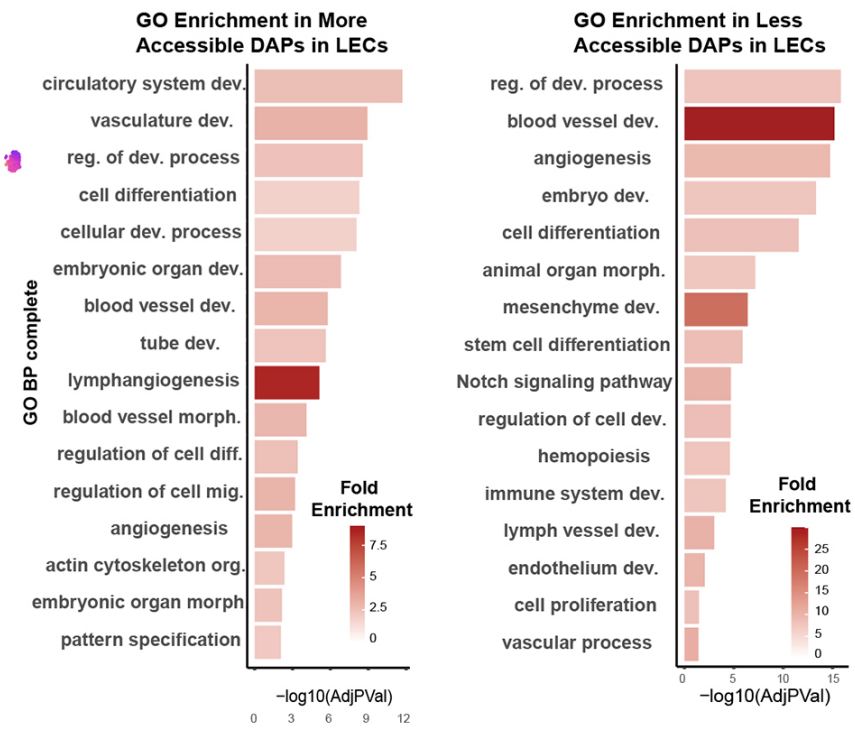
(E) Confocal projections of the heart at 5 dpf labelled with *Tg(-2.1prox1a: basEGFP; ACry:GFP)*^{uu10kk} (cyan) and *Tg(kdr-l:ras-cherry)*^{s916} (magenta). A: atrium. V: ventricle. BA: bulbus arteriosus. Arrowheads: cardiac valves position.

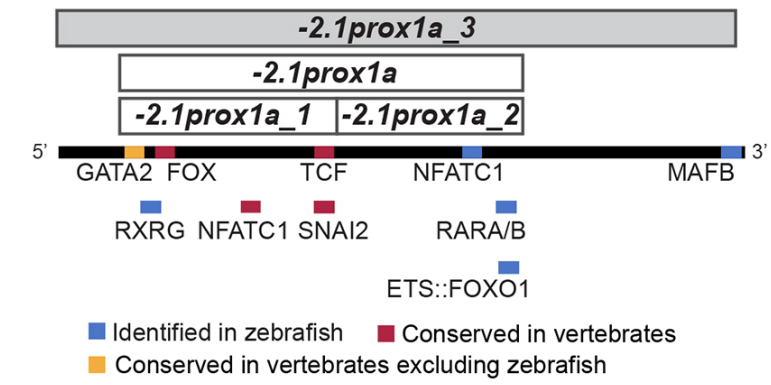
(F) Conserved motif structure of the identified lymphatic -2.*1prox1a* enhancer. The motif sequences are listed in Table S4.

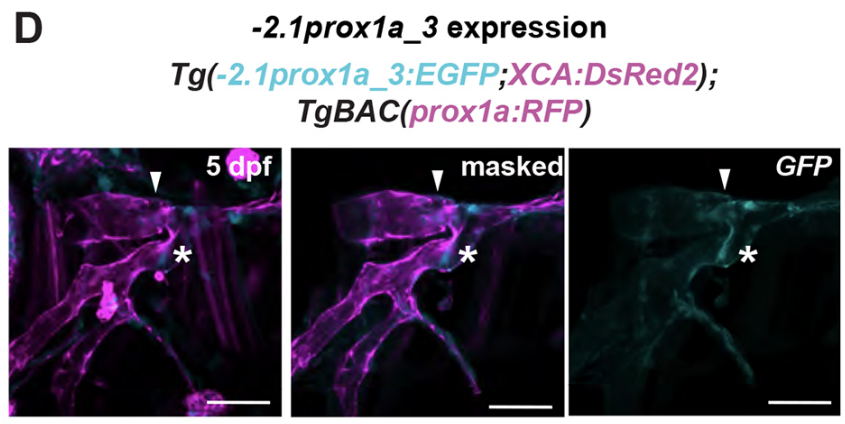
(G) Predicted conserved binding site for *-2.1prox1a* in vertebrates. Motif locations are listed in Table S4.

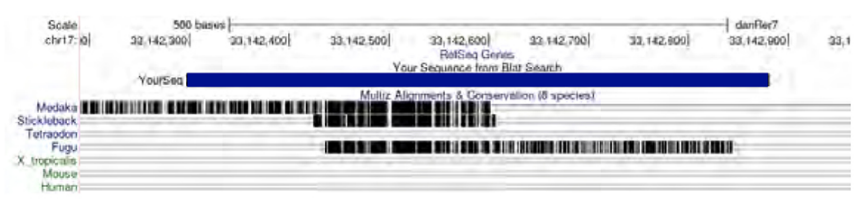
Scales bars represent 50µm.

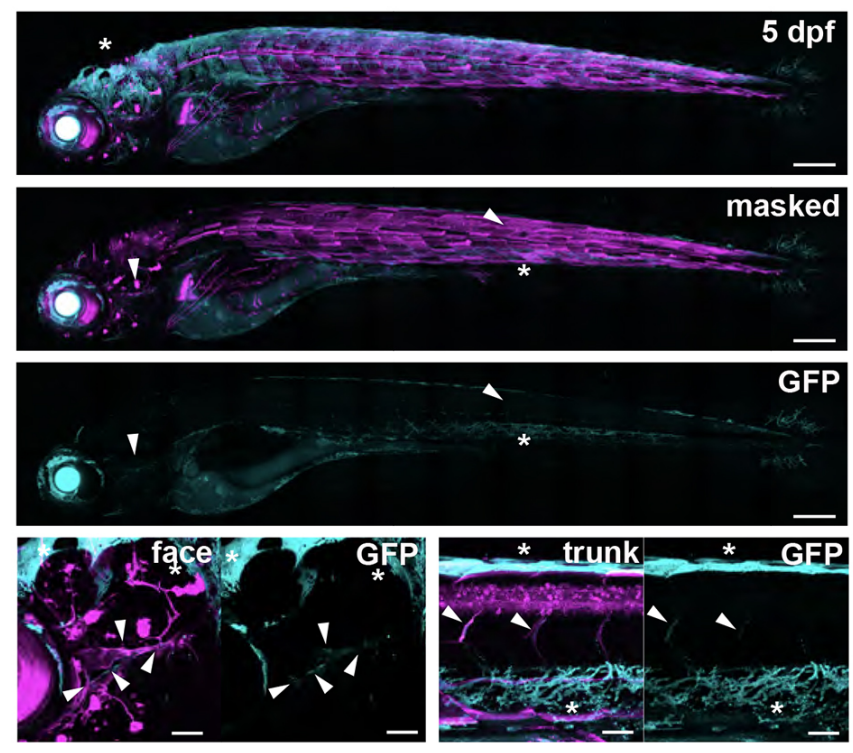


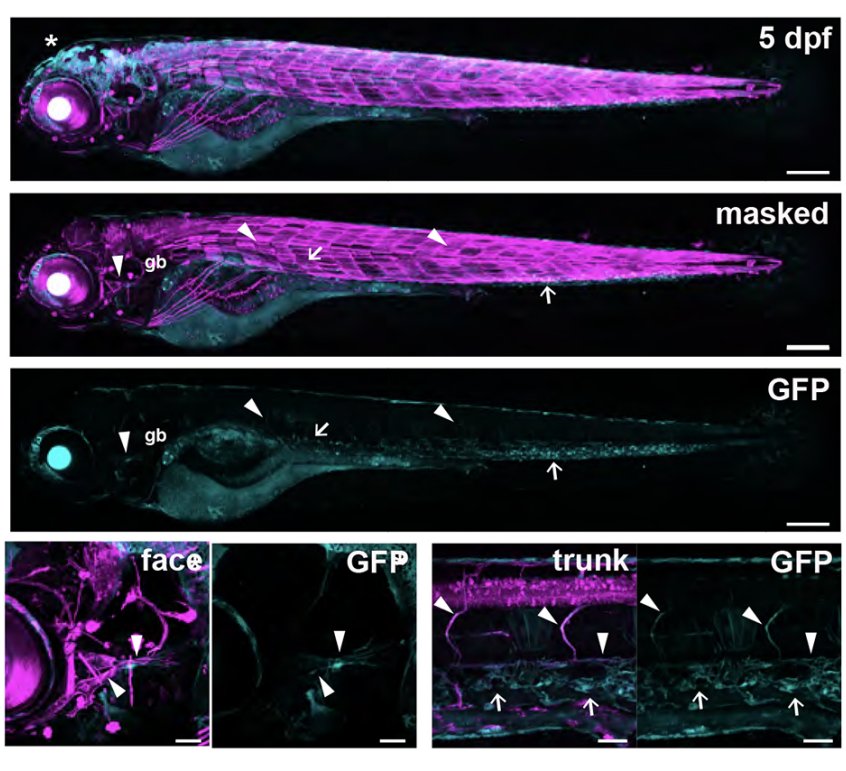












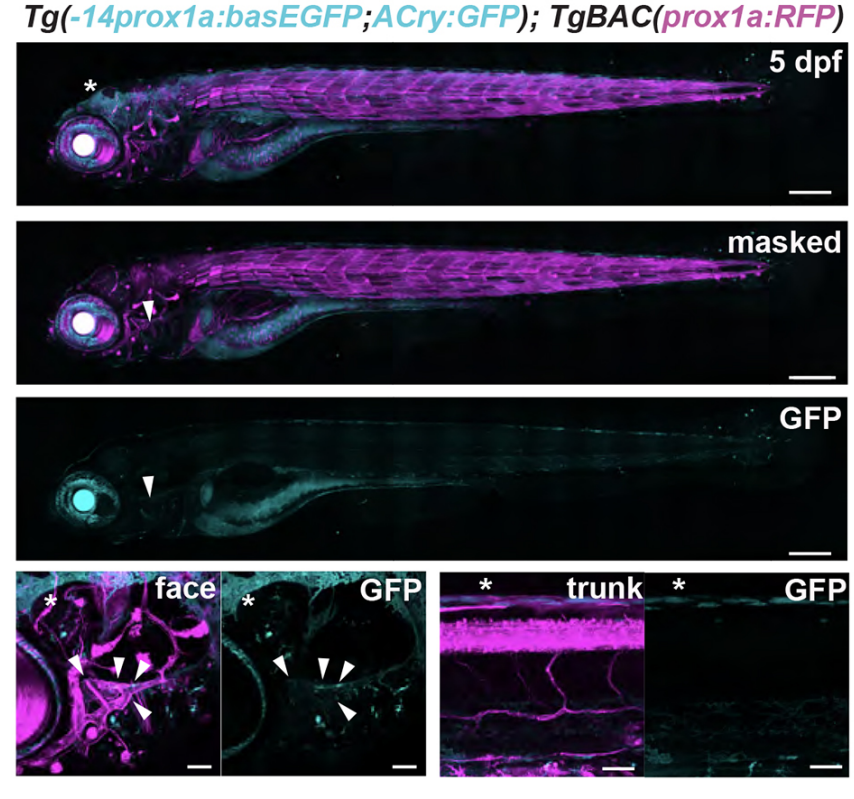


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Fig. S3. snATAC-seq validation and additional ATAC-identified enhancers characterisation

(A) Clusters of endothelial cells (ECs) and endocardium at 4 dpf with umaps showing the chromatin state at known lymphatics and venous loci. High accessibility at *prox1a* and *cdh6* marks the lymphatic endothelial cell (LECs) cluster. High *kdrl* accessibility marks the venous endothelial cells (VECs) and arterial endothelial cell (AECs) cluster, and high *lyve1b* accessibility marks LECs and VECs. muLECs: Mural lymphatic endothelial cells.

(B) GO enrichment of developmentally relevant terms in differentially accessible peaks in LECs.

(C) Schematic representation of the *-2.1prox1a* enhancer indicating the overlap between the *-2.1prox1a_3* sequence from the identified peak using the ATAC-sequencing, *-2.1prox1a* identified using the conservation method (Fig. 1A) and the segmentation into *-2.1prox1a*_1 and *-2.1prox1a*_2 (Fig. 4B). Predicted binding for lymphatic-associated transcription factors is indicated below the enhancer fragments. Blue: Binding sites identified in zebrafish (p-val < 1e- 04). Red: Conserved binding sites within vertebrates. Yellow: Binding sites conserved in vertebrates but absent in zebrafish.

(D) Confocal projections of facial lymphatics labelled with *Tg(-2.1prox1a_3:EGFP; XCA:DsRed2)*^{uom119} (cyan) and *Tg(prox1a:RFP)*^{nim5} (magenta) at 5 dpf. Arrowheads: Expression in the developing lymphatic valve. Asterisks: expression in the facial lymphatic endothelium. Scale bars: 50µm.

(E) UCSC Genome browser Multiz alignment of the *-71prox1a* enhancer in zebrafish, showing conservation in Actinopterygii but not tetrapods.

(F-H) Tile-scan, face and trunk confocal projection of whole F1 embryo at 5dpf. In second and third panels, skin signal has been removed manually. Tile-scan scalebar = 200µm, zoom-in panels scalebar: 50µm.

(F) Embryo labelled with *Tg(-87prox1a:basEGFP;ACry:GFP)*^{uu14kk} (cyan) and *Tg(prox1a:RFP)*^{nim5} (magenta). Arrowhead: expression in the facial lymphatics. Asterisks: expression in the skin and connective tissue.

(G) Embryo labelled with *Tg(-71prox1a:basEGFP;ACry:GFP)*^{uu15kk} (cyan) and *Tg(prox1a:RFP)*^{nim5} (magenta). Arrowhead: expression in the lymphatics. Asterisks: expression in the skin. Arrows: expression in the PCV. gb: expression in the gallbladder.

(H) Embryo labelled with *Tg(-14prox1a:basEGFP;ACry:GFP)*^{uu16kk} (cyan) and *Tg(prox1a:RFP)*^{nim5} (magenta). Arrowhead: expression in the facial lymphatics. Asterisks: expression in the skin.

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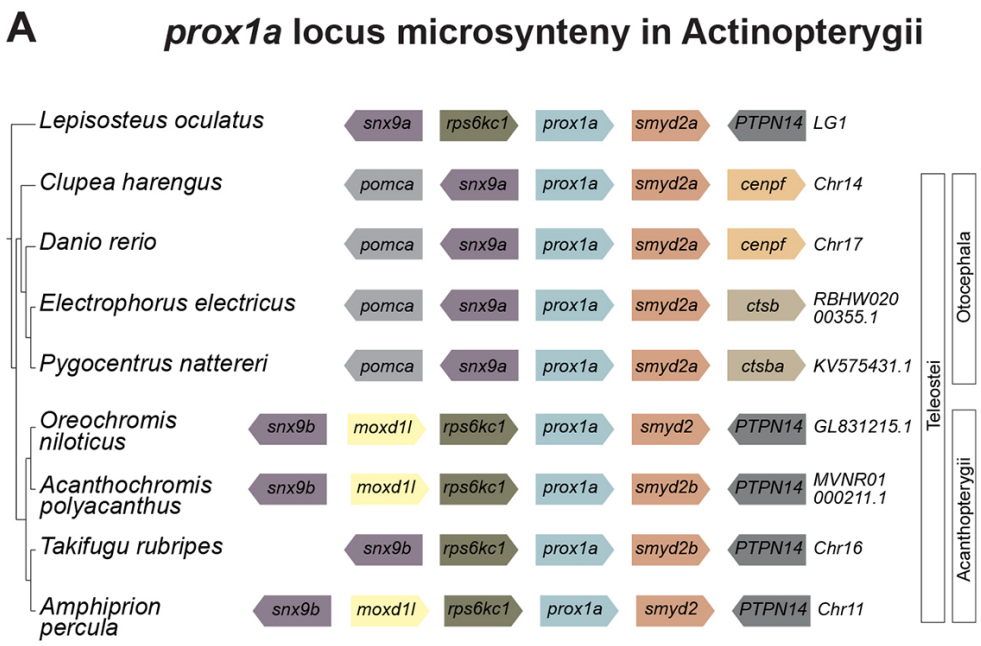

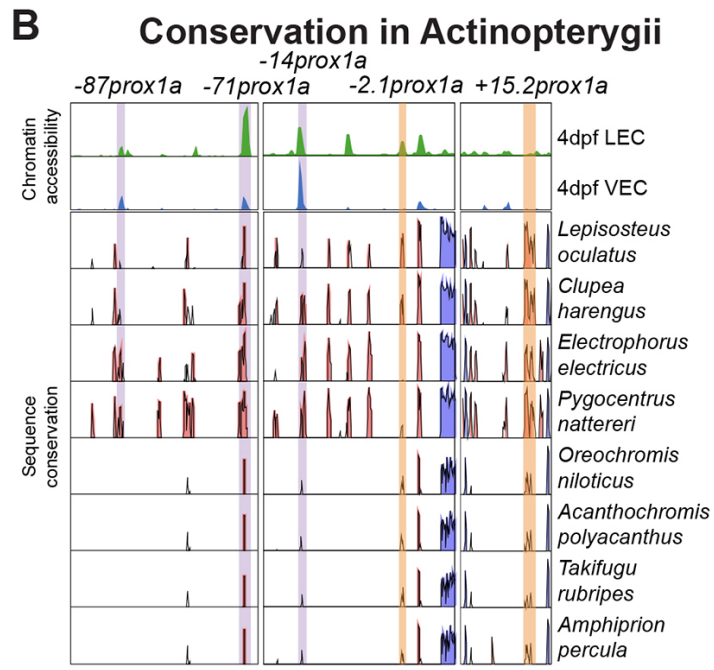

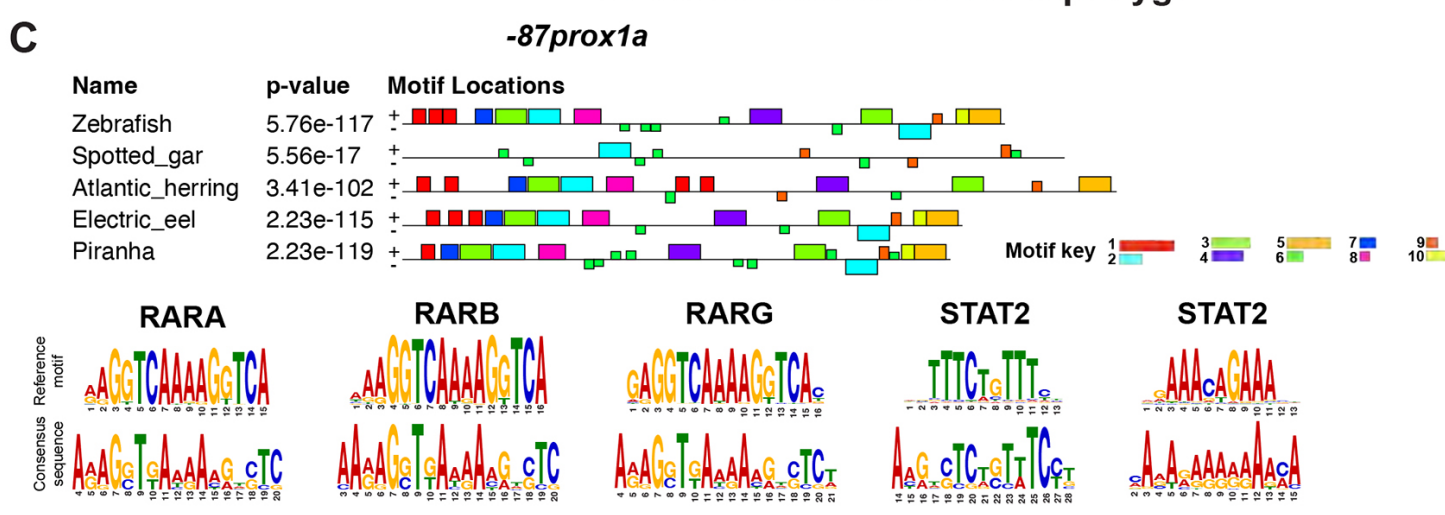

Development • Supplementary information

Fig. S4. *prox1a* microsynteny in Actinopterygii and predicted TFs binding to the snATAC-identified enhancers

(A) The microsynteny surrounding the Actinopterygii *prox1a* locus. Downstream of *prox1a*, *Smyd2a* is found in all analysed sequences. Upstream of *Prox1/prox1a, Rps6ck1* is found in *Lepisosteus oculatus* and Euteleostei, but not in Otocephala. The annotations used are listed in Table 1.

(B) Conservation analysis of the identified *prox1a* enhancers 9 Actinopterygii species. Results are shown compared to zebrafish. Blue peaks: exons; Red peaks: conserved non-coding DNA; Purple bars: snATAC-identified enhancers; Orange bar: conservation-identified enhances.

(C) Conserved motif structure and predicted conserved binding sites of the identified lymphatic *-87prox1a* enhancer in Actinopterygii. Motif locations are listed in Table S4 and S5.

(D) Conserved motif structure and predicted conserved binding sites of the identified lymphatic *-71prox1a* enhancer in Actinopterygii. Motif locations are listed in Table S4 and S5.

(E) Conserved motif structure and predicted conserved binding sites of the identified lymphatic *-14prox1a* enhancer in Actinopterygii. Motif locations are listed in Table S4 and S5.

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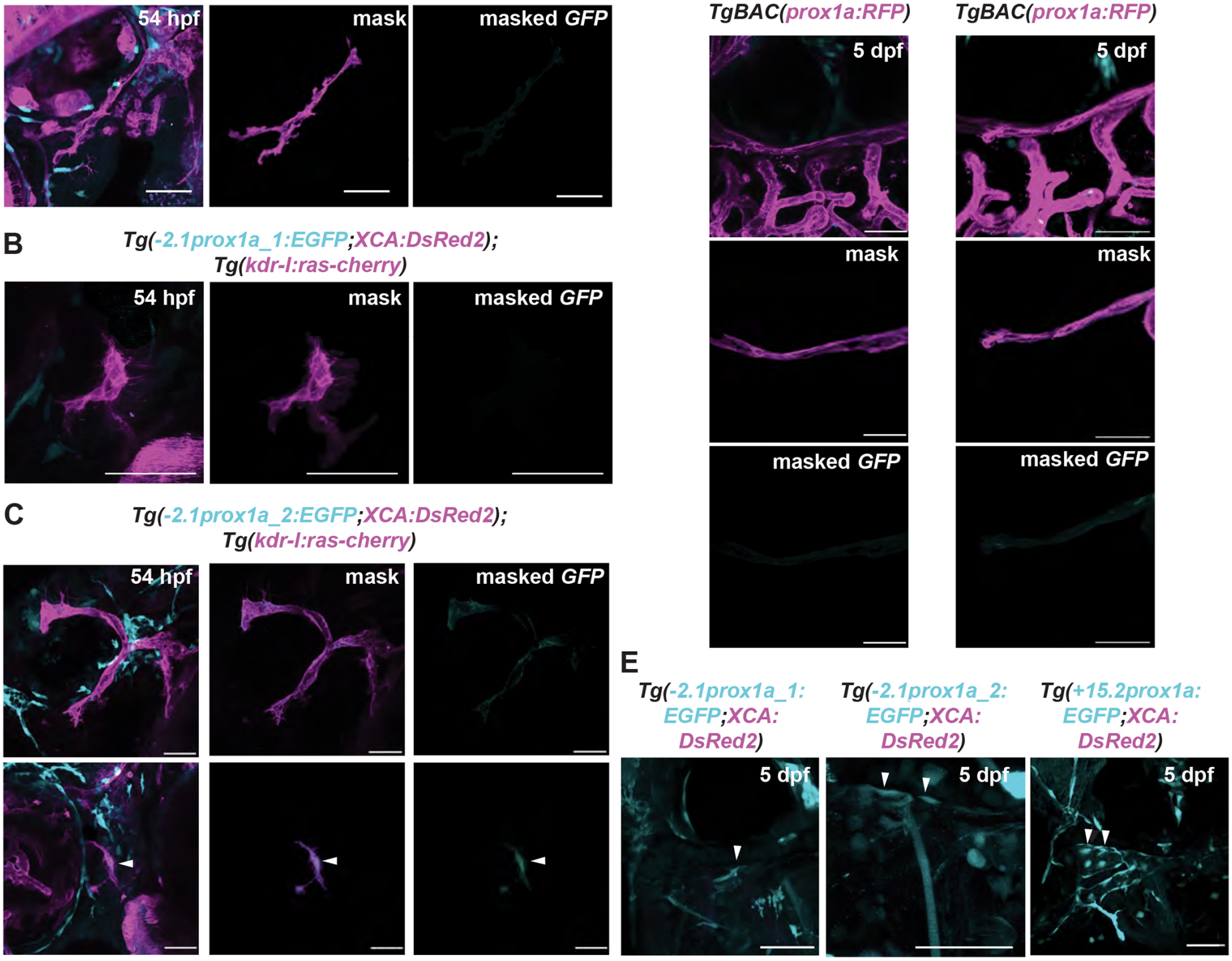

Fig. S5. Additional characterisation of the core element of *-2.1prox1a* activity

(A) Confocal projections of facial lymphatics labelled with *Tg(-2.1prox1a_1:EGFP; XCA:DsRed2)*^{uu5kk} (cyan) and *Tg(prox1a:RFP)*^{nim5} (magenta) at 54 hpf, showing no lymphatic expression.

(B) Confocal projections of VA-L labelled with *Tg(-2.1prox1a_1:EGFP;XCA:DsRed2)*^{uu5kk} (cyan) and *Tg(kdr-l:ras-cherry)*^{s916} (magenta) at 54 hpf, showing no lymphatic expression.

(C) Confocal projections of facial lymphatics labelled with *Tg(- 2.1prox1a_2:EGFP; XCA:DsRed2)*^{uu6kk} (cyan) and *Tg(kdr-l:ras-cherry)*^{s916} (magenta) at 54 hpf, showing -2.*1prox1a_2* driven expression in the VA-L (arrowhead).

(D) Confocal projections of the primary head sinus labelled with *Tg(- 2.1prox1a_1:EGFP; XCA:DsRed2)*^{uu5kk} and *Tg(-2.1prox1a_2:EGFP;XCA:DsRed2)*^{uu6kk} at 5 dpf, showing no expression.

(E) Enhancer-driven expression in *Tg(-2.1prox1a_1:EGFP;XCA:DsRed2)*^{uu5kk}, *Tg(- 2.1prox1a_2:EGFP;XCA:DsRed2)*^{uu6kk} and *Tg(+15.2prox1a:EGFP;XCA:DsRed2)*^{uu7k} in the facial lymphatics (arrowheads) at 5 dpf, excluding bleed-through as a source of the signal. Scales bars represent 50µm.

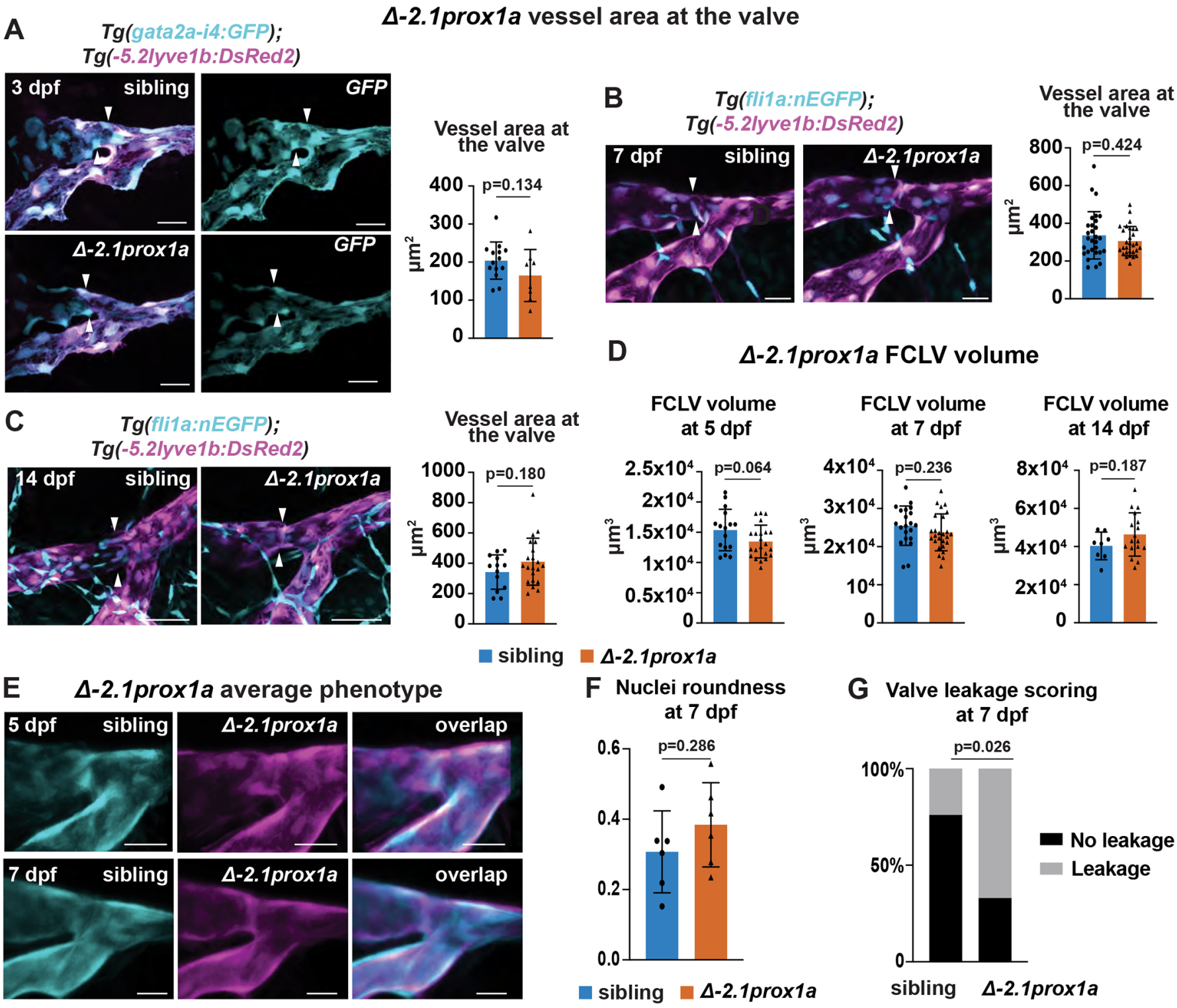
A
Tg(gata2a-i4:GFP);
Tg(-5.2lyve1b:DsRed2)
Δ-2.1prox1a vessel area at the valve
3 dpf
sibling
GFP
Δ-2.1prox1a
GFP
Vessel area at the valve
p=0.134
µm²
400
300
200
100
0
B
Tg(fli1a:nEGFP);
Tg(-5.2lyve1b:DsRed2)
7 dpf
sibling
Δ-2.1prox1a
Vessel area at the valve
p=0.424
µm²
800
600
400
200
0
C
Tg(fli1a:nEGFP);
Tg(-5.2lyve1b:DsRed2)
14 dpf
sibling
Δ-2.1prox1a
Vessel area at the valve
p=0.180
µm²
1000
800
600
400
200
0
D
Δ-2.1prox1a FCLV volume
FCLV volume at 5 dpf
p=0.064
µm³
2.5x10⁴
2x10⁴
1.5x10⁴
1x10⁴
0.5x10⁴
0
FCLV volume at 7 dpf
p=0.236
µm³
4x10⁴
3x10⁴
2x10⁴
10⁴
0
FCLV volume at 14 dpf
p=0.187
µm³
8x10⁴
6x10⁴
4x10⁴
2x10⁴
0
sibling
Δ-2.1prox1a
E
Δ-2.1prox1a average phenotype
5 dpf
sibling
Δ-2.1prox1a
overlap
7 dpf
sibling
Δ-2.1prox1a
overlap
F
Nuclei roundness at 7 dpf
p=0.286
0.6
0.4
0.2
0.0
sibling
Δ-2.1prox1a
G
Valve leakage scoring at 7 dpf
p=0.026
100%
50%
0
No leakage
Leakage
sibling
Δ-2.1prox1a

Fig. S6. Additional characterisation of Δ-*2.1prox1a* mutant embryos

(A) Confocal projections of *Tg(gata-i4:GFP)*^{uu11kk} *(cyan)*and *Tg(-5.2lyve1b:DsRed2)*^{nz101} (magenta) in sibling and Δ-*2.1prox1a* mutant embryos at 3 dpf. Quantification of vessel section at the valve (arrowheads) in Δ-*2.1prox1a* embryos at 3 dpf. Scale bar: 20µm. Mean ± sd, sibling (n=14) vs mutants (n=8): two-tailed Student's t-test, ns (p=0.134). 2 technical replicates, biological replicates correspond to the number of datapoints per condition.

(B) Confocal projections of *Tg(fli1:nEGFP)*^{y7} *(cyan)*and *Tg(-5.2lyve1b:DsRed2)*^{nz101} (magenta) in sibling and Δ-*2.1prox1a* mutant embryos at 7 dpf. Quantification of vessel section at the valve (arrowheads) in Δ-*2.1prox1a* embryos at 7 dpf. Scale bar: 50µm. Mean ± sd, sibling (n=29) vs mutants (n=29), two-tailed Mann-Whitney test, ns (p=0.424). 3 technical replicates, biological replicates correspond to the number of datapoints per condition.

(C) Confocal projections of *Tg(fli1:nEGFP)*^{y7} *(cyan)*and *Tg(-5.2lyve1b:DsRed2)*^{nz101} (magenta) in sibling and Δ-*2.1prox1a* mutant embryos at 14 dpf. Quantification of vessel section at the valve (arrowheads) in Δ-*2.1prox1a* embryos at 14 dpf. Scale bar: 50µm. Mean ± sd, sibling (n=13) vs mutants (n=21): two-tailed Student's t-test, ns (p=0.180). 3 technical replicates, biological replicates correspond to the number of datapoints per condition.

(D) Quantification of FCLV volume in Δ-*2.1prox1a* embryos at 5, 7 and 14 dpf. Mean ± sd. Left: two-tailed Student's t-test of 5 dpf siblings (n=16) vs Δ-*2.1prox1a* (n=22), ns (p=0.064). Centre: two-tailed Student's t-test of 7 dpf siblings (n=21) vs Δ-*2.1prox1a* (n=26), ns (p=0.236). Right: two-tailed Student's t-test of 14dpf siblings (n=8) vs Δ-*2.1prox1a* (n=17), ns (p=0.187). (E) Average valve phenotype in sibling (cyan) vs Δ-*2.1prox1a* (magenta) embryos at 5 dpf and 7 dpf, showing similar gross vessel morphology. Scale bar: 20µm.

(F) Quantification of sibling (n=6) vs Δ-*2.1prox1a* mutant (n=6) valve cell nuclei roundness in TEM-imaged embryos at 7 dpf. Mean ± sd. Representative images are present in Fig. 5D. two-tailed Student's t-test, ns (p=0.286). 2 technical replicates, biological replicates correspond to the number of datapoints per condition.

(G) Scoring of valve leakage in Qtracker injected 7 dpf siblings (n=21) vs Δ-*2.1prox1a* (n=12) embryos. Mean ± sd. Representative images are present in Fig. 5E. Fisher's test, p=0.026. 2 technical replicates, biological replicates correspond to the number of datapoints per condition.

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Table S1. mVista analysis sequences

Available for download at
https://journals.biologists.com/dev/article-lookup/doi/10.1242/dev.202525#supplementary-data

Table S2. Tested *prox1a* putative CREs

Available for download at
https://journals.biologists.com/dev/article-lookup/doi/10.1242/dev.202525#supplementary-data

Table S3. Primers and gRNAs

Available for download at
https://journals.biologists.com/dev/article-lookup/doi/10.1242/dev.202525#supplementary-data

Table S4. Conserved motifs detected in evolutionary conserved enhancers

Available for download at
https://journals.biologists.com/dev/article-lookup/doi/10.1242/dev.202525#supplementary-data

Table S5. Predicted TFs binding sites in *prox1a* enhancers

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Table S6. Complete list motifs based on zebrafish (p-val < 1e-04)

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Table S7. GO terms enrichment in more and less accessible sequences in LECs

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Table S8. gRNAs used

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Additional files are uploaded containing tables separately

Table S5A. Predicted TFs binding sites in *prox1a* enhancers

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https://journals.biologists.com/dev/article-lookup/doi/10.1242/dev.202525#supplementary-data

Table S6A. Complete list motifs based on zebrafish (p-val < 1e-04)

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Table S7A. GO terms enrichment in more and less accessible sequences in LECs

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