Figures and figure supplements

Yap1 promotes sprouting and proliferation of lymphatic progenitors downstream of Vegfc in the zebrafish trunk

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Figure 1. Nuclear EGFP-YAP changes dynamically in the developing trunk lymphatic vasculature. (A–B) The TEAD reporter line [Tg(fli1:Gal4db-TEAD2ΔN-2A-mC);(UAS:GFP)] shows Yap1 activity in vasculature, parachordal LECs (PLs) and cardinal vein sprouts (arrowhead) of the 2 dpf trunk (A) as well as in the thoracic duct (TD) at 5 dpf (B). Scale bars: 40 μm. (C) Maximum projection of 8 PLs in a two dpf embryo showing EGFP-YAP in green [Tg(fli1:EGFP-YAP)] and the nucleus in red (C'), [Tg(fli1:H2B-mCherry)] and merge (C''). Scale bar: 25 μm. (D–E) High power single z-sections of selected PLs from C, showing nuclear YAP in PL2 and PL3, but low nuclear YAP in PL4 (D–D') and PL7 (E–E'). Scale bars: 10 μm. (F) Quantification of nuclear EGFP/mCherry average pixel intensity across individual PLs from multiple embryos at 2 dpf. Each bar represents a single PL (n = 47), each grey shade a different embryo (n = 5). PLs in (C and D) highlighted in the green box. EGFP/mCherry Ratios have been calculated using mean fluorescent intensities in 3D. (G) Scatter Plot of the Nuclear EGFP/mCherry average pixel intensity for individual PLs (n = 5 embryos). Each colour indicates PLs from a different embryo. Values calculated in 3D measurements of the mean fluorescent intensity for EGFP and mCherry (0.66 ± 0.04, n = 47). (H) Pearson Correlation Plot of the Nuclear EGFP/mCherry Ratio values in F and the Nuclear/Cytoplasmic EGFP Ratio values in Figure 1—figure supplement 1B (r = 0.52, 95% confidence intervals: 0.27 to 0.70, R square = 0.27, p=0.0002(***)). The two distinct approaches produce correlative measurements. (I) Average nuclear pixel intensity graphs from cell tracks of single PLs time-lapse imaged from 2 to 3 dpf. EGFP-YAP intensity (green) is compared over time with H2B-mCherry (red) intensity in individual nuclei. Arrow points to a cell division during time-lapse.

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Figure 1—figure supplement 1. Measurements of EGFP-YAP intensity in lymphatic progenitor nuclei over time. (A) Nuclear EGFP/mCherry average pixel intensity ratios measured manually on a single z-plane at the centre of the nucleus in 2D. Same PLs as in Figure 1F. (B) Nuclear/cytoplasmic EGFP.
average pixel intensity ratio measured manually on a single z-stack at the centre of the nucleus using the same approach. A. Same PLs as in Figure 1F. (C) Scatter Plot of the Nuclear/Cytoplasmic EGFP average pixel intensity measured as a ratio across individual PLs (1.81 ± 0.11, n = 47) from multiple embryos (n = 5). Each colour indicates PLs from a different embryo. Values above one correspond to PLs with more EGFP-YAP in the nucleus while values below one correspond to PLs with more EGFP-YAP in the cytoplasm. (D) Scatter Plot of the fluorescent intensity values calculated manually in 2D on a single z-stack image for each PL selecting the centre of the nucleus (0.58 ± 0.04, n = 47). (E) Pearson Correlation comparing the similarity between the automated 3D- analysis and the manual 2D- analysis of the nuclear EGFP/mCherry mean intensity ratios. The values from both analyses show a strong correlation (r = 0.69, confidence intervals: 0.50 to 0.81, R squared = 0.47, p<0.0001 (***)). (F) Nuclear EGFP/mCherry ratios of single PLs (n = 6) tracked in 90 min high speed spinning disc time-lapse Videos (see Supplementary Video No 2). (F’–F”) Mean nuclear EGFP and mCherry intensities of single PLs from time-lapse Videos. Nuclear EGFP-YAP intensity remains relatively stable over the 90 min time interval (z-stack acquired every 1 min). The slow decrease in fluorescent intensity is due to signal bleaching. (G) Further examples of relative fluorescent intensity graphs generated from PL nuclei followed from 2 to 3 dpf in time-lapse Videos. High EGFP intensity corresponds to high Yap1 protein abundance in the nucleus (boxed area). H2B-mCherry fluorescence highlights the stable concentration of this control fluorophore in PL nuclei over time. Both fluorophores driven by the same promoter.

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Figure 2. Yap1 acts cell autonomously to control trunk lymphangiogenesis in zebrafish. (A) Overall morphology of sibling (left) and MZyap1<sup>tn101-/-</sup> mutant (right) at 5 dpf. Arrowheads indicate mild craniofacial defects and absent swim bladder. Scale bar 200 μm. (B–C) Trunk vasculature of sibling and 

Figure 2 continued on next page.
MZyap1ncv101-/mutant embryos at 5 dpf. Veins and lymphatics are displayed in white [Tg(dab2b:EGFP)], erythrocytes show normal blood flow in red [Tg(gata1:DsRed)]. Asterisks mark absent lymphatic vessels. (C) Trunk vasculature of sibling and MZyap1mw48-/mutant at 6 dpf. Vascular nuclei are marked in green [Tgf(fl1a:nEGFP)], venous and lymphatic vessels in white [Tg(−5.2lyve1b:DsRed)]. Asterisks indicate absent lymphatic vessels. Scale bars: 50 µm in B and C. (D) Percentage of TD fragments formed per somite, scored across six somites in total for siblings and MZyap1ncv101-/mutants at 5 dpf (sibling: 100% ± 0, n = 23, MZyap1ncv101-/: 49% ± 7.33, n = 22, p=0.0001). (E) Quantification of the total number of LECs across 6 somites at 6 dpf (sibling: 37 ± 1.91, n = 11, MZyap1ncv101-/: 8 ± 3.79, p<0.0001). (F) Schematic showing the cell transplantation technique. Blastomere cells are transplanted from donor (EGFP) into host (mCherry) embryos. This results in a chimeric host embryo (right) with randomly located, transplanted EC grafts. (G) Representative images of host chimeric trunk vessels at 5 dpf. Wildtype (wt) donor ECs contribute to all vascular EC types, while MZyap1ncv101-/ mutant ECs show reduced propensity to contribute to lymphatic structures. Asterisk marks missing TD. Scale bars: 50 µm. (H) Graft sizes analysed for vascular grafts. Numbers within bars represent the number of embryos scored for each graft size. (I) Percentage of embryos with EC grafts contributing to arterial (wt into wt: 96% ± 4; MZyap1ncv101-/ into wt: 100% ± 0; p=0.31, not significant (ns)), venous (wt into wt: 91% ± 6, MZyap1ncv101-/ into wt: 67% ± 10, p=0.04 (**)), and lymphatic vessels (wt into wt: 43% ± 10, MZyap1ncv101-/ into wt: 8% ± 6, p=0.005 (**)). Wildtype into wildtype total number of EC grafts: n = 23; MZyap1ncv101-/ mutant into wildtype total number of EC grafts: n = 24. Dorsal aorta (DA); Posterior Cardinal Vein (PCV); Thoracic Duct (TD); Intersegmental Lymphatic vessel (ISLV), Dorsal Longitudinal Lymphatic vessel (DLLV). DOI: https://doi.org/10.7554/eLife.42881.009

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Figure 2—figure supplement 1. \( \text{Zyap}^{1-/-} \) mutants only exhibit mild lymphatic defects in the trunk and \( \text{MZyap}^{1-/-} \) mutants form facial lymphatics. (A) Trunk lymphatics of sibling and \( \text{Zyap}^{1-/-} \) mutant at 5 dpf. The vasculature is shown in red \( \text{Tg(kdrl:mCherry)} \), venous and lymphatics in green \( \text{Tg(dab2b:EGFP)} \). Scale bar: 50 \( \mu \text{m} \). (B) Heads of siblings and \( \text{MZyap}^{1-/-} \) mutants showing facial lymphatics including the lateral facial lymphatic (LFL), medial facial lymphatic (MFL), otolithic lymphatic vessel (OLV), branchial arch lymphatics (LAA), and developing lymphatic loop (LL). Scale bar: 50 \( \mu \text{m} \). (C) Percentage of thoracic duct (TD) segments formed across 6 somites at 5 dpf in siblings \( (98.17 \pm 1.83, n = 18) \) and \( \text{Zyap}^{1-/-} \) mutants \( (77.54 \pm 10.14, n = 13; p=0.0269) \). (D) Score of facial lymphatics in siblings \( (LL: 0.75 \pm 0.08, n = 10) \) and \( \text{MZyap}^{1-/-} \) mutants \( (LL: 0.67 \pm 0.11, n = 6; p=0.55 \text{ (ns)}) \). Each point represents the vessel score for one embryo. Matrix scores for LFL, MFL and OLF between sibling and \( \text{MZyap}^{1-/-} \) are identical, thus not significantly different.

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**Figure 2—figure supplement 2.** MZyap1+/− mutants do not show major defects in blood vessel formation. (A) Blood vasculature (EC nuclei labelled in green) of sibling and MZyap1mw48+/− mutant at 24 hpf. S1-4 indicate the quantified sprouts of the sibling and M1-4 the equivalent for the MZyap1mw48+/−.
Figure 2—figure supplement 2 continued

mutant in D–F). Dorsal aorta (DA). Scale bars in A–C: 50 μm. (B) Blood vasculature of [Tg(fli1a:nEGFP)] sibling and MZ yap1<sup>mut</sup> mutant at 32 hpf. Posterior cardinal vein (PCV). (C) Trunk vasculature of sibling and MZ yap1<sup>mut</sup> mutant at 50 hpf showing normal lumen formation in white ([Tg(kdrl::GFP)]). Missing parachordal LECs (PLs) are marked by asterisks. (D) Quantification of number of nuclei for all sprouts (S1-S4 for sibling, M1-4 for MZ yap1<sup>mut</sup>) at 24 hpf (sibling: 2.73 ± 0.12, n = 56; MZ yap1<sup>mut</sup>: 2.14 ± 0.13, n = 56; p=0.0013 (**)) and 32 hpf (sibling: 2.93 ± 0.09, n = 44; MZ yap1<sup>mut</sup>: 2.91 ± 0.15, n = 44; p=0.90 (ns)). (E) Sprout length for all sprouts in μm (S1-S4 for sibling, M1-4 for MZ yap1<sup>mut</sup>) at 24 hpf (sibling: 50.91 ± 1.70, n = 56; MZ yap1<sup>mut</sup>: 44.74 ± 2.32, n = 56; p=0.034 (*) and 32 hpf (sibling: 85.57 ± 2.98, n = 44; MZ yap1<sup>mut</sup>: 74.57 ± 3, n = 44; p=0.01 (*)). (F) Quantification of nuclei number in single sprouts at 24 hpf (S1: 2.64 ± 0.20, n = 14, M1: 2.64 ± 0.23, n = 14, p=0.99 (ns); S2: 2.64 ± 0.19, n = 14, M2: 2.07 ± 0.25, n = 14, p=0.08 (ns); S3: 2.93 ± 0.37, n = 14, M3: 2.00 ± 0.28, n = 14, p=0.05 (ns); S4: 2.71 ± 0.16, n = 14, M4: 1.86 ± 0.27, n = 14, p=0.01 (*) and 32 hpf (S1: 3.27 ± 0.14, n = 11, M1: 2.64 ± 0.24, n = 11, p=0.04 (*); S2: 3.09 ± 0.16, n = 11, M2: 2.91 ± 0.31, n = 11, p=0.61 (ns); S3: 2.63 ± 0.15, n = 11, M3: 3.18 ± 0.23, n = 11, p=0.06 (ns); S4: 2.73 ± 0.20, n = 11, M4: 2.91 ± 0.39, n = 11, p=0.68 (ns)).

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Figure 3. MZ Yap1f/f mutants display defects in LEC numbers but not specification. (A) Trunk vasculature (EC nuclei in green, veins and lymphatics in white) of sibling and MZ yap1mww48/mww48 mutant at 2 dpf. Arrowheads indicate posterior cardinal vein (PCV) sprouts. Asterisks mark absent parachordal LECs.
Figure 3 continued

(PLs). Dorsal aorta (DA). Scale bars: 50 μm. (B) Total number of lyve1-positive ECs departing the PCV across 6 somites at 2 dpf. (C) Number of endothelial cells in venous intersegmental vessels (vISV) across 6 somites at 2 dpf (sibling: 13 ± 0.94, n = 14; MZyap1<sup>-/-</sup>: 12 ± 1.33, n = 14; p=0.24 (ns)).

(D) Number of PLs scored across 6 somites at 2 dpf (sibling: 3 ± 0.56, n = 14; MZyap1<sup>-/-</sup>: 0.36 ± 0.23, n = 14; p<0.0001 (***)). (E) Number of PLs scored across 6 somites at 3 dpf (sibling: 4.5 ± 0.20, n = 14; MZyap1<sup>-/-</sup>: 3.31 ± 0.36, n = 13; p=0.0075(**)). (F) Immunofluorescence staining for EC nuclei (green) and Prox1 (red) in sibling and MZyap<sup>tm1mod<sup>/-</sup> mutants at 36 hpf. Arrows point to Prox1<sup>+</sup> LEC progenitors. Scale bars: 30 μm. (G) Quantification of Prox1<sup>+</sup> cells in PCV and CV sprouts scored across 6 somites at 36 hpf (sibling: 3.47 ± 0.65, n = 19; MZyap1<sup>-/-</sup>: 3.31 ± 0.61, n = 16; p=0.86 (ns)). (H) Maximum projection stills from time-lapse Videos from 32 to 65 hpf. Sibling still images show normal lymphangiogenesis with PCV sprouts, PL formation, sprout detachment and PL proliferation (upper panels). MZyap<sup>tm1mod<sup>/-</sup> mutant one displays abnormal sprouting and looping of PCV sprouts that are retained until the end of the Video (central panels). MZyap<sup>tm1mod<sup>/-</sup> mutant two also exhibits abnormal sprouting and PCV loop formation but also forms PLs (lower panels). Scale bars: 25 μm. Timelapse imaging began at 32 hpf.

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Figure 4. Yap1 mediates endothelial cell proliferation downstream of Vegfc. (A) Trunk vasculature at 3 dpf displaying endothelial cells (ECs) in green from uninjected, p53 morpholino (MO) injected and p53 + yap1 MO injected embryos. Asterisks highlight missing parachordal LECs (PLs). Scale bars: 50 μm.

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µm. (B) Trunk vasculature at 3 dpf from uninjected, p53 MO injected and p53 +yap1 MOs injected embryos of the Tg(prox1a:KaiT4xUAS:uncTagRFP); Tg(10xUAS:vegfc) strain. yap1 MO injection rescues the EC proliferation phenotype. ECs in green. Asterisks mark missing PLs. Scale bars: 50 µm. (C) Quantification of total EC number across four somites in vegfc-unstimulated (uninjected: 343 ± 9, n = 18; p53 MO: 341 ± 9, n = 18; p53 +yap1 MOs: 248 ± 15, n = 18; p<0.0001(****)) and vegfc-stimulated embryos (uninjected: 811 ± 35, n = 18; p53 MO: 794 ± 38, n = 18, p53 +yap1 MOs: 212 ± 10, n = 18; p<0.0001(****)). (D) Schematic representation of vegfc-overexpressing cell transplantations for single muscle grafts in [Tg(fli1a:nEGFP); (#C0 5.2lyve1b:DsRed2)] hosts of siblings and MZ yap1mew8/-. (E) Schematic of transplanted host embryo at 3 dpf. Muscle grafts produce excessive Vegfc causing a hyperproliferation response in adjacent PLs. (F) Quantification of PL number within one somite responding to the vegfc-OE single muscle graft at 3 dpf (sibling: 23.00 ± 1.19, n = 18; MZ yap1mew8/-: 6.36 ± 0.71, n = 22; p<0.0001 (***)). (G) Examples of 3 different vegfc-OE muscle grafts (false coloured) in siblings (left panels) and MZ yap1mew8/- mutants (right panels). Merge images show ECs in green, lymphatic and venous ECs are red (yellow) [Tg(fli1a:nEGFP); (#C0 5.2lyve1b:DsRed2)]. Scale bars: 25 µm.

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Figure 5. Vegfc promotes nuclear Yap1 in developing lymphatic progenitors. (A) Schematic showing the transplantation of vegfc-OE cells into EGFP-YAP reporter hosts. (B) Trunk vasculature of YAP reporter host embryos with neuron and muscle grafts expressing RFP (magenta) at 56 hpf. Control...
Figure 5 continued

grafts (left) and vegfc-OE grafts (right). Posterior cardinal vein (PCV); Muscle (M); parachordal LECs (PLs); Neuron (N). Scale bars: 50 μm in B and C. (C) Heatmaps of maximum projections of EGFP-YAP from B showing PLs and PCV. Red corresponds to high EGFP-YAP fluorescence. (C’) Lower panels show heatmaps of EGFP-YAP in PL nuclei. (D) EGFP intensity for each PL expressed as a ratio to the average of 6 dorsal aorta (DA) cells (unresponsive to vegfc). six embryos per group: untransplanted host (control) (PL n = 27), transplanted control without the UAS:vegfc construct (TP Control) (PL n = 40) and vegfc-OE transplanted embryos (TP vegfc-OE) (PL n = 251). Individual embryos indicated by an individual grey shade. (E) Heatmaps of EGFP-YAP depicting PL nuclei of embryos at 66 hpf that were treated with DMSO (left) and SL327 for 13 hr (hrs) (right). Max projections are from time-lapse Videos. Scale bars: 30 μM. (F) EGFP mean intensity for each PL/DA in panel D (Control: 1.61 ± 0.13, n = 27, TP Control: 1.93 ± 0.12, n = 40, p=0.08 (ns), TP vegfc-OE: 3.14 ± 0.07, n = 251; p<0.0001(****)). (G) Quantification of PL number across six somites for each group (Control: 5 ± 0.26, n = 6, TP Control: 8 ± 0.78, n = 16; p=0.0157 (*), TP vegfc-OE: 44 ± 8.04, n = 16; p=0.0001(**)). (H) Quantifications of the mean EGFP/mCherry fluorescent intensity ratio in PL nuclei from time-lapse Video stills. Embryos were mounted in DMSO as control (n = 5) or SL327 (15 μM) (n = 5) and continuously imaged for 12 hr starting at 54 hpf (1 hr drug treatment), finishing at 66 hpf (13 hr) (1 hr DMSO: 0.33 ± 0.02, n = 20; 1 hr SL327: 0.24 ± 0.02, n = 23; p=0.0036 (**)) (13 hr DMSO: 0.39 ± 0.02, n = 20; 13 hr SL327: 0.26 ± 0.03, n = 16; p=0.0006(**)). (I) Quantifications of mean EGFP/mCherry Ratio in PLs of embryos at 66 hpf treated with DMSO/SL327 for 12 hr from 54 hpf without time-lapse imaging (12 hr DMSO: 0.47 ± 0.02, PLs n = 43, 13 hr SL327: 0.37 ± 0.03, PLs n = 22; p=0.02 (*)). (J) Quantification of PL number in embryos treated with DMSO/SL327 for 12 hr at 66 hpf without time-lapse imaging (12 hr DMSO: 8.6 ± 1.08, embryo n = 5, 13 hr SL327: 4.4 ± 0.81, embryo n = 5; p=0.014 (*)).

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Figure 5—figure supplement 1. The autonomous EGFP-YAP response to Vegfc in transplanted ECs. (A) Schematic displaying the transplantation of \( [Tg(fli1a:EGFP-YAP);fli1a:H2B-mCherry] \) endothelial cells (ECs) into control (no aberrant vegfc) and vegfc-overexpression (OE) host embryos at 3–4 hpf. Figure 5—figure supplement 1 continued on next page
Figure 5—figure supplement 1 continued

(B) Average Nuclear EGFP Intensity as ratio of vegfc-responsive cells (parachordal LECs (PLs) and venous ECs (VECs))/vegfc unresponsive dorsal aorta (DA) cells. Grafted ECs in the TP vegfc-OE hosts show a higher amount of nuclear EGFP-YAP compared to the TP Control (TP Control: 1.52 ± 0.07, n = 52; TP vegfc-OE: 2.51 ± 0.14, n = 52; p=0.0001(****)). (C–D) Maximum projections of transplanted EGFP-YAP EC grafts in control hosts (C) and vegfc-OE hosts (D) at 2 dpf. Scale bars: 25 μm. (C’) shows the EGFP fluorescence as heatmap of the maximum projections for TP control and (D’) for TP vegfc-OE (red indicates high EGFP fluorescence) (central panels). (C”) and (D”) display nuclear EGFP-YAP in PCV and PL ECs as a heatmap (right panels). (E) Nuclear EGFP average pixel intensity as a ratio of vegfc-responsive cells (PLs and VECs) and vegfc-unresponsive cells (DA cells) for EC grafts in TP control (n = 7) and TP vegfc-OE host embryos (n = 6). Each grey shade represents a different embryo, each bar shows the EGFP-YAP fluorescent ratio of a vegfc-responsive cell nucleus. Statistics are shown in B.

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