PNAS
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Supplementary Information for

Vegfaa instructs cardiac muscle hyperplasia in adult zebrafish

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www.pnas.org/cgi/doi/10.1073/pnas.1722594115

SI MATERIALS AND METHODS

Zebrafish. Wild-type or transgenic zebrafish of the EK/AB strain were used for all experiments. *βactin2:loxp-mCherry-STOP-loxp-DTA*, *βactin2:loxp-mTagBFP-STOP-loxp-nrg1; cmlc2:CreER*, *gata4*:*eGFP*, *LENP2:EGFP, tcf21*:*DsRed, fli1a:eGFP, kdrl:DsRed;* and *cmlc2:H2A-EGP,* transgenic fish have been described (1-8). Resection of ~20% of the cardiac ventricular apex was performed as described previously (9). To induce expression of *nrg1* in CMs, adult *cmlc2:CreER; βact2:BS-nrg1* were treated for 24 hours with 5 μM tamoxifen. To induce ablation of CMs, adult *ZCAT* were treated for 24 hours with 1 μM tamoxifen. For larval experiments, *cmlc2:CreER; βact2:BS-vegfaa; cmlc2:H2A-EGFP* fish were treated with 10 μM 4-hydroxytamoxifen for 6 hours at 2 dpf. Embryos were then placed in egg water containing 0.1% DMSO, or 10μm DAPT (Selleckchem) until 7 dpf as previously described (10, 11). Procedures involving animals were approved by the Institutional Animal Care and Use Committee at Duke University.

Generation of Transgenic Zebrafish. To generate *βact2:BS-vegfaa*, *vegfaa* cDNA was ligated into the AgeI/NotI site of the *βact2:loxP-TagBFP-STOP-lox-P* vector (12). To generate *vegfaa:EGFP* fish, the translational start codon of *vegfaa* in the BAC clone CH211-169N14 (BACPAC Resources Center) was replaced with the EGFP sequence by Red/ET recombineering technology (GeneBridges) as described in reference (6). The full names for these transgenic strains are *TgBAC(vegfaa:EGFP)*^{pd260} and *Tg(βactin2:loxP-mTagBFP-STOP-loxP-vegfaa)*^{pd262}. To induce recombination in adult fish, *cmlc2:CreER*; *βact2:BS-vegfaa* fish were bathed in 5 μM tamoxifen (Sigma) for 24 hours.

Histologic Analysis and Imaging. Probes for *vegfaa*, *raldh2, snai2, twist1, pdgfrb, notch1b, and deltaC* were generated from 6 dpf zebrafish cDNA by using the primer sequences *twist1b* forward: GTTTGAGCTACGCGTTCTCG; *twist1b* reverse: TGCACAGGATTCGAACTAGAGG; *snai2* forward: TTAGAGCAGATCAGCGCTGG; *snai2* reverse: CTTCCCAATCGGAGGCACTT; *pdgfrb* forward: GTCCAGTGGAGGAAAGACGG; *pdgfrb* reverse: ACATCAGCGCTTGTGTCTCA. In situ hybridization probes were prepared and hybridizations were performed with the aid of an InSituPro robot (Intavis) as described (1). Primary and secondary antibody staining for immunofluorescence was performed as described in (6). Antibodies used in this study were anti-PCNA (mouse; Sigma) at 1:200, anti-Mef2 (rabbit; Santa Cruz Biotechnology) at 1:75, CT3 (mouse; DSHB) at 1:150, anti-EGFP (rabbit; Abcam) at 1:200, Alexa Fluor 488 (mouse and rabbit; Life Technologies) at 1:200, Alexa Fluor 594 (mouse and rabbit; Life Technologies) at 1:200, and Alexa Fluor 633 (mouse; Life Technologies) at 1:200. Confocal imaging was performed using a Zeiss LSM 700 or a Zeiss LSM 510 microscope. Thickness of the compact muscle layer was determined by staining hearts with anti-Tnnt antibody, selecting the largest section, and performing tilescan confocal imaging. For each section, the primordial muscle layer was used to define the anatomic boundary of the compact muscle and the inner trabecular muscle. The primordial muscle layer can be visualized as a thin stripe with increased fluorescence with either BFP or after immunostaining for Tnnt. Maximal thickness was determined by taking the largest distance from the cortical muscle layer to the epicardial surface of Tnnt signal. Measurements were performed on blinded images using ImageJ (2). An analogous approach was used to quantify maximal *tcf21*+ cell thickness from sections of hearts from *tcf21:DsRed* fish. Cardiomyocyte proliferation indices were obtained from confocal images of sections that were stained for Mef2 and PCNA. The resulting images were blinded, cropped to the resection plane, manually thresholded using ImageJ, and

subjected to segmentation analysis using the EBImage package of R/Bioconductor (13). The proliferation index is the number of $Mef2^+/PCNA^+$ nuclei over the total number of $Mef2^+$ nuclei (2, 4, 6, 12, 14-16). Blinded regenerates were scored on a scale from 1 to 3 after staining for Tnnt as described in reference (17). Images from *cmlc2:H2A-EGFP* fish were quantified by blinding the images and manually thresholding the images using the "Spots" feature of the Imaris software package (Bitplane). Picro-Mallory staining was performed as described previously and imaging was performed using a Zeiss AxioImager.Z2 microscope (18, 19).

RNA-Seq. RNA was extracted by using whole ventricles from *cmlc2:CreER*; *βact2:BS-vegfaa* fish as previously described (15). RNA libraries were generated and sequencing was performed through the Duke Sequencing and Genome Technologies Shared Resource. RNA-seq data was processed using the TrimGalore toolkit which employs Cutadapt to trim low quality bases and Illumina sequencing adapters from the 3' end of the reads (20, 21). Only reads that were 20nt or longer after trimming were kept for further analysis. Reads were mapped to the GRCz10v87 version of the zebrafish genome and transcriptome using the STAR RNA-seq alignment tool. Reads were kept for subsequent analysis if they mapped to a single genomic location (22, 23). Gene counts were compiled using the HTSeq tool (24). Only genes that had at least 10 reads in any given library were used in subsequent analysis. Normalization and differential expression was carried out using the DESeq2 Bioconductor package with the R statistical programming environment (25-27). The false discovery rate was calculated to control for multiple hypothesis testing. Gene set enrichment analysis was performed to identify differentially regulated gene ontology terms for each of the comparisons performed (28).

SUPPLEMENTARY FIGURES

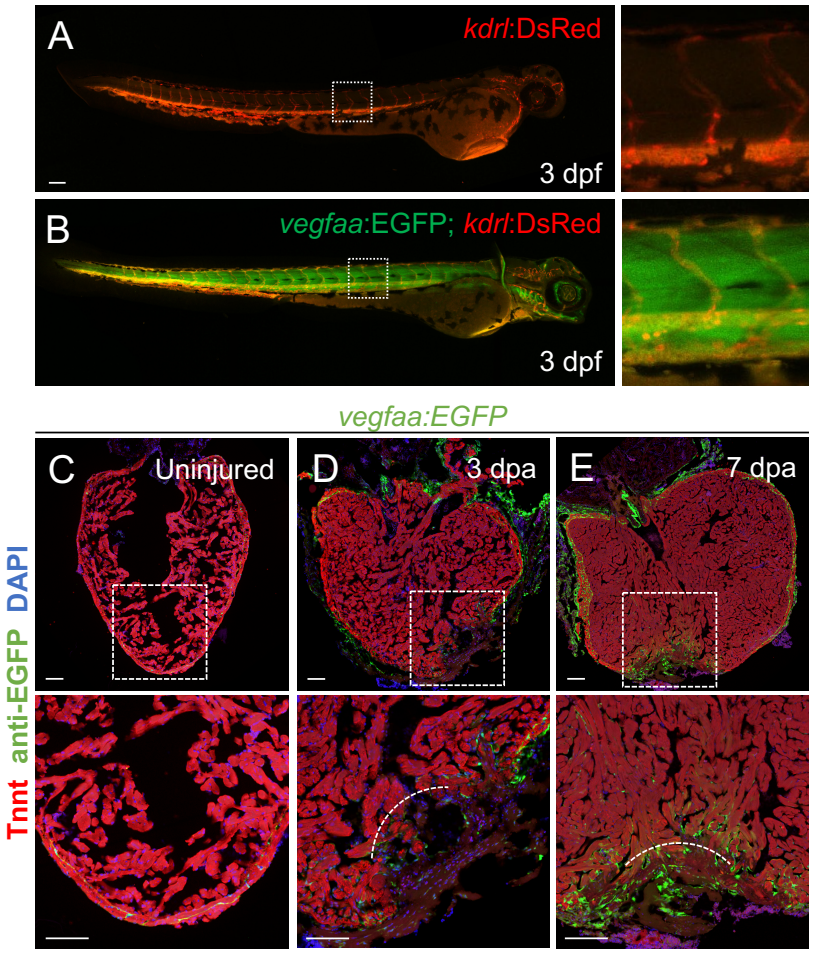

Figure S1. *vegfaa:EGFP* expression in larval and adult fish. (A-B) Tile-scanned, maximum intensity projection of the trunk of a *kdrl:DsRred* fish and a *vegfaa:EGFP*; *kdrl:DsRred* fish at 3 dpf. Boxed region corresponds to the magnified region shown in the adjacent panel. (C-E) Tiled images of *vegfaa:EGFP* hearts without injury, at 3 days post amputation (dpa), and at 7 dpa. Sections were immunostained with for EGFP and Tnnt. Boxed region corresponds to the magnified region shown in the adjacent panel. Curved lines approximate the amputation plane. (Scale bars 100 μm)

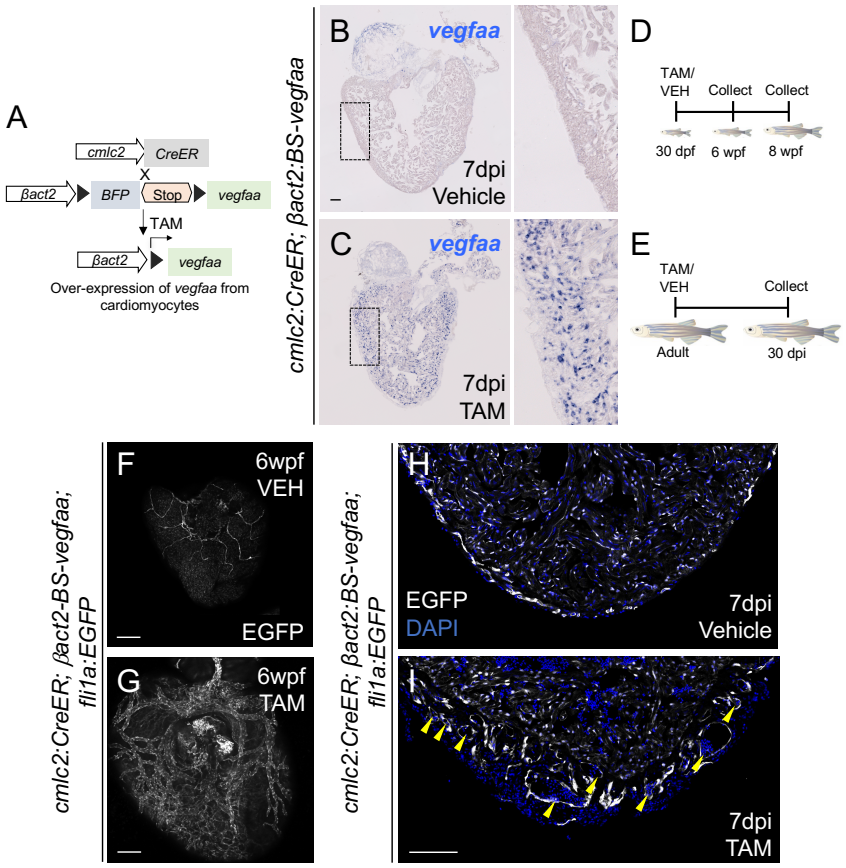

Figure S2. Transgenic overexpression of *vegfaa* from cardiomyocytes. (A) Transgenic approach to overexpress *vegfaa* from cardiomyocytes. (B-C) In situ hybridization for *vegfaa* on sections from ventricles of *cmlc2*:*CreER*; *βact2:BS-vegfaa* animals 7 days post incubation (dpi) with vehicle or tamoxifen. Violet staining indicates expression. Boxed region corresponds to the magnified region shown in the adjacent panels. (D-E) Schematic depicting juvenile and adult experiments in Figure 3. (F-G) Tile-scanned images of ventricular surfaces from *cmlc2:CreER; βact2:BS-vegfaa; fli1a:EGFP* fish at 6 wpf following incubation with vehicle or tamoxifen at 30 dpf. (H-I) Tile-scanned images of sections from ventricles of *cmlc2*:*CreER*; *βact2:BS-vegfaa; fli1a:EGFP* animals 7 days after treatment with vehicle or tamoxifen. Yellow arrows indicate nucleated blood cells in the lumen of ectopic blood vessels. (Scale bars 100 μm)

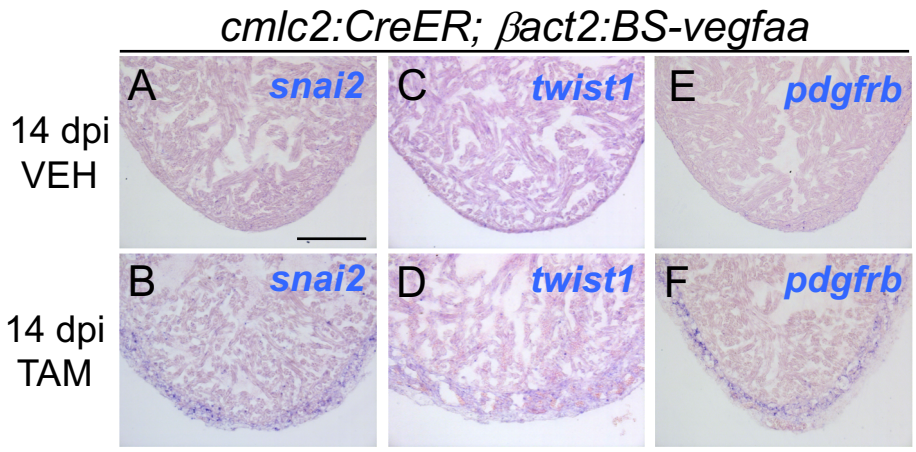

Figure S3. Cardiac *vegfaa* overexpression induces markers of EMT. In situ hybridization for *snai2 (A-B), twist1 (C-D), and pdgfrb (E-F)* on sections from ventricles of *cmlc2*:*CreER*; *βact2:BS-vegfaa* animals 14 days after treatment with vehicle or tamoxifen. Violet staining indicates expression. (Scale bars 100 μm)

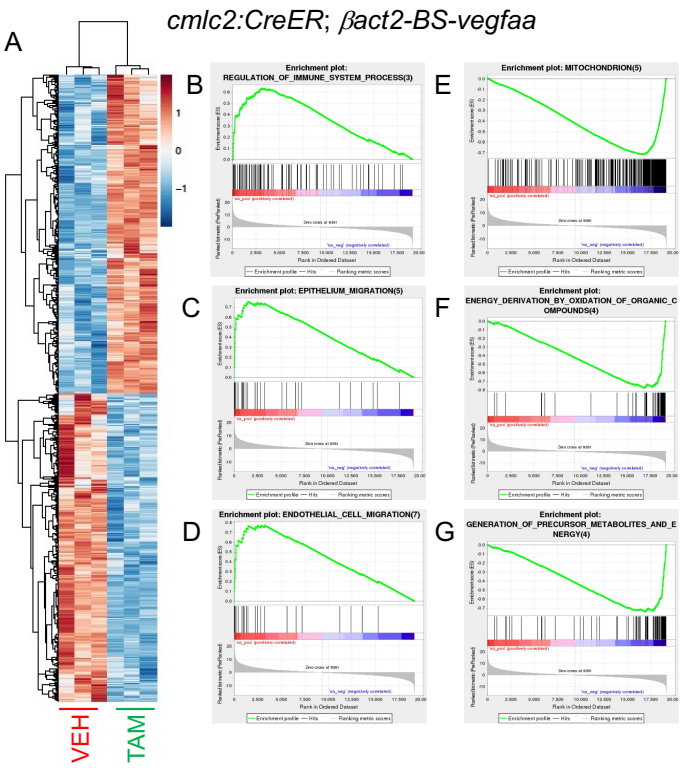

Figure S4: Transcriptional profiles of *vegfaa* overexpression. (A) Heatmap of RNA-seq data for *cmlc2*:*CreER*; *βact2:BS-vegfaa* hearts 14 days after treatment with vehicle (n = 3) or tamoxifen (n = 3). (B-D) Selected results for pathways enriched in *vegfaa* overexpression hearts as determined by Gene Set Enrichment Analysis (GSEA). (E-G) Selected results for pathways downregulated in *vegfaa* overexpression hearts as determined by GSEA.

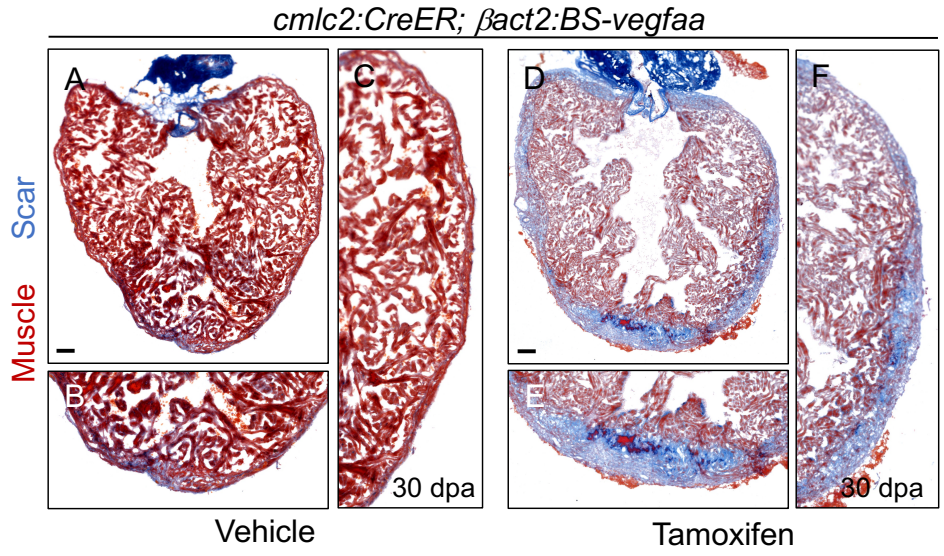

Figure S5. Cardiac *vegfaa* overexpression causing scarring after ventricular resection. Picro-Mallory staining of regenerating hearts overexpressing *vegfaa*. Tiled, brightfield images of sections from control animals show limited collagen (blue) at the site of injury and within the ventricular wall (A-C). By contrast, animals overexpressing vegfaa have scar at the resection site and throughout the ventricular wall (D-F). (Scale bars 100 μm)

Additional Dataset S1 (separate file). Differentially expressed transcripts following *vegfaa* overexpression. RNA-Seq was performed using *cmlc2*:*CreER*; *βact2:BS-vegfaa* hearts 14 dpi with vehicle (n = 3) or tamoxifen (n = 3). Gene ID corresponds to the Ensembl ID of the mapped transcript. logFC is provided for tamoxifen treated hearts relative to vehicle treated hearts. p-value is the result of an unadjusted t-test. p_{adj} is the FDR corrected p-value.

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