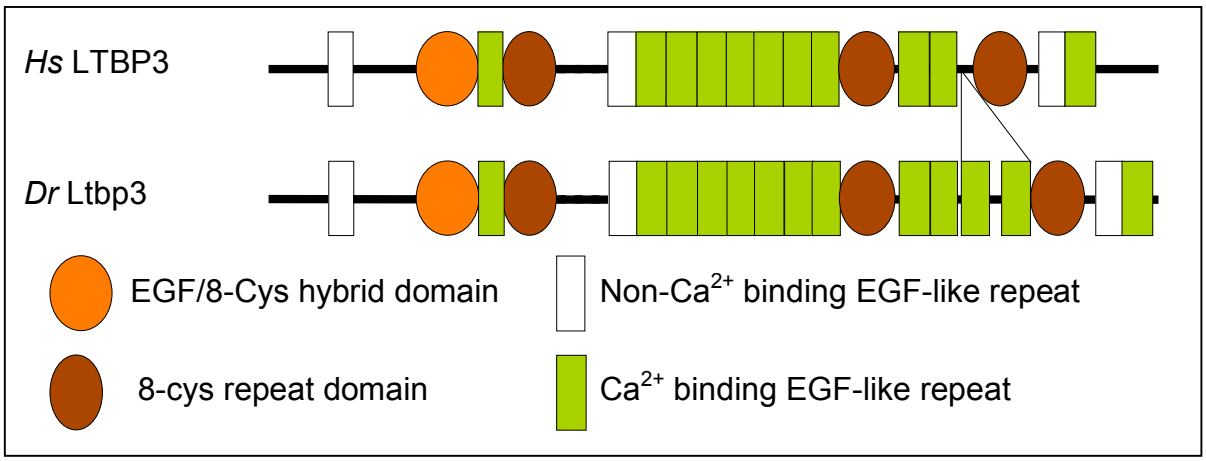
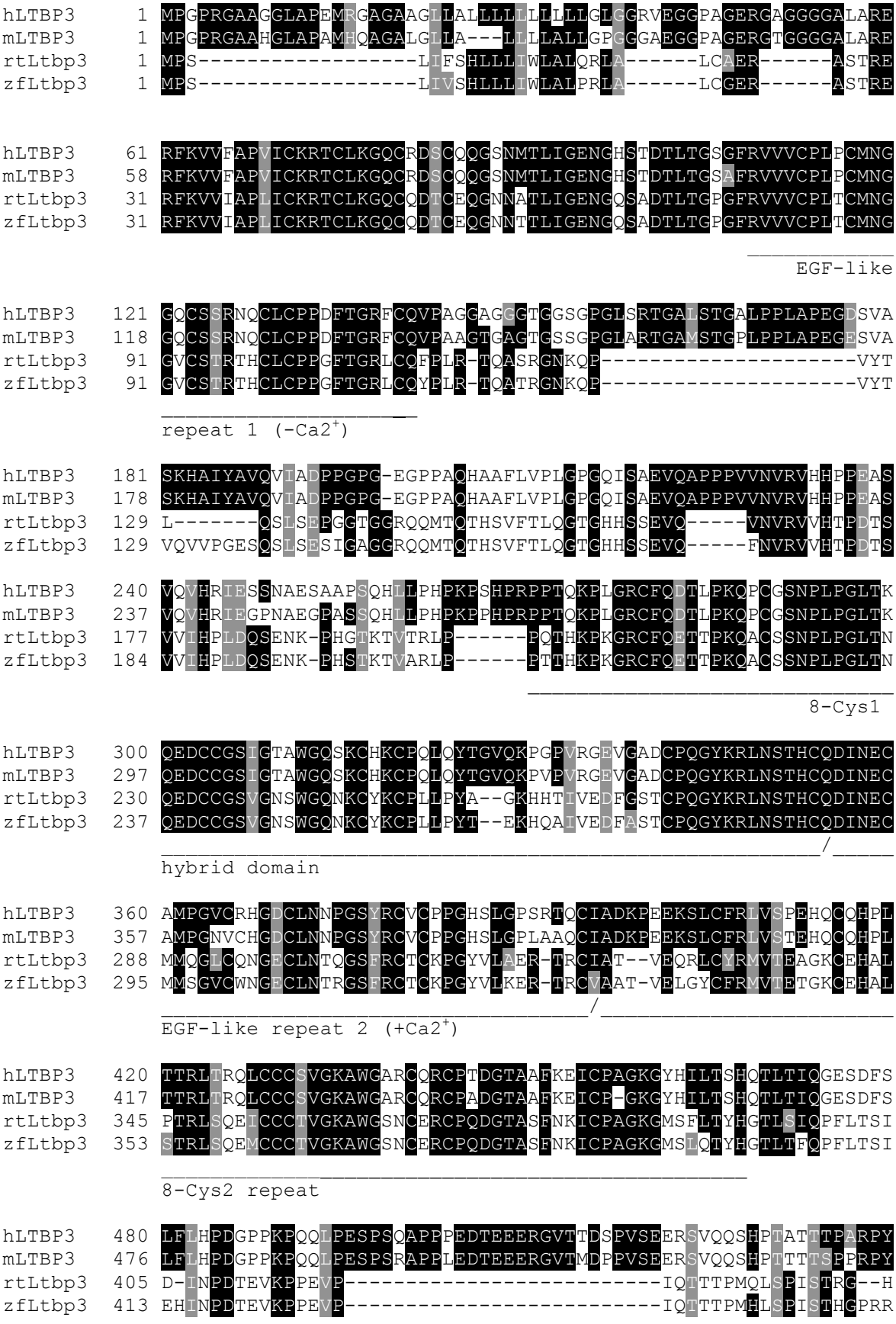
a.



Supplementary Figure 1 – Zebrafish Ltbp3 domain structure and alignments. a, Schematic diagram comparing the domain structures of human (*Hs*; *Homo sapiens*) and zebrafish (*Dr*; *Danio rerio*) LTBP3 proteins. **b,** Alignment of LTBP3 proteins from human (h), mouse (m), rainbow trout (rt), and zebrafish (zf) (see below). Domains in zebrafish Ltbp3 shown in (**a**) are underlined. **c,** Alignment of the 16 EGF-like domains in zebrafish Ltbp3 (see below). Asterisks (*) mark shared cysteine residues. **d,** Alignment of the EGF-like/8-cys hybrid and 8-cys repeat domains in zebrafish Ltbp3 (see below). Asterisks (*) mark shared cysteine residues.

b.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zfLtbp3 1 MPS-----------------LIVSHLLLIWLALPRLA------LCGER------ASTRE
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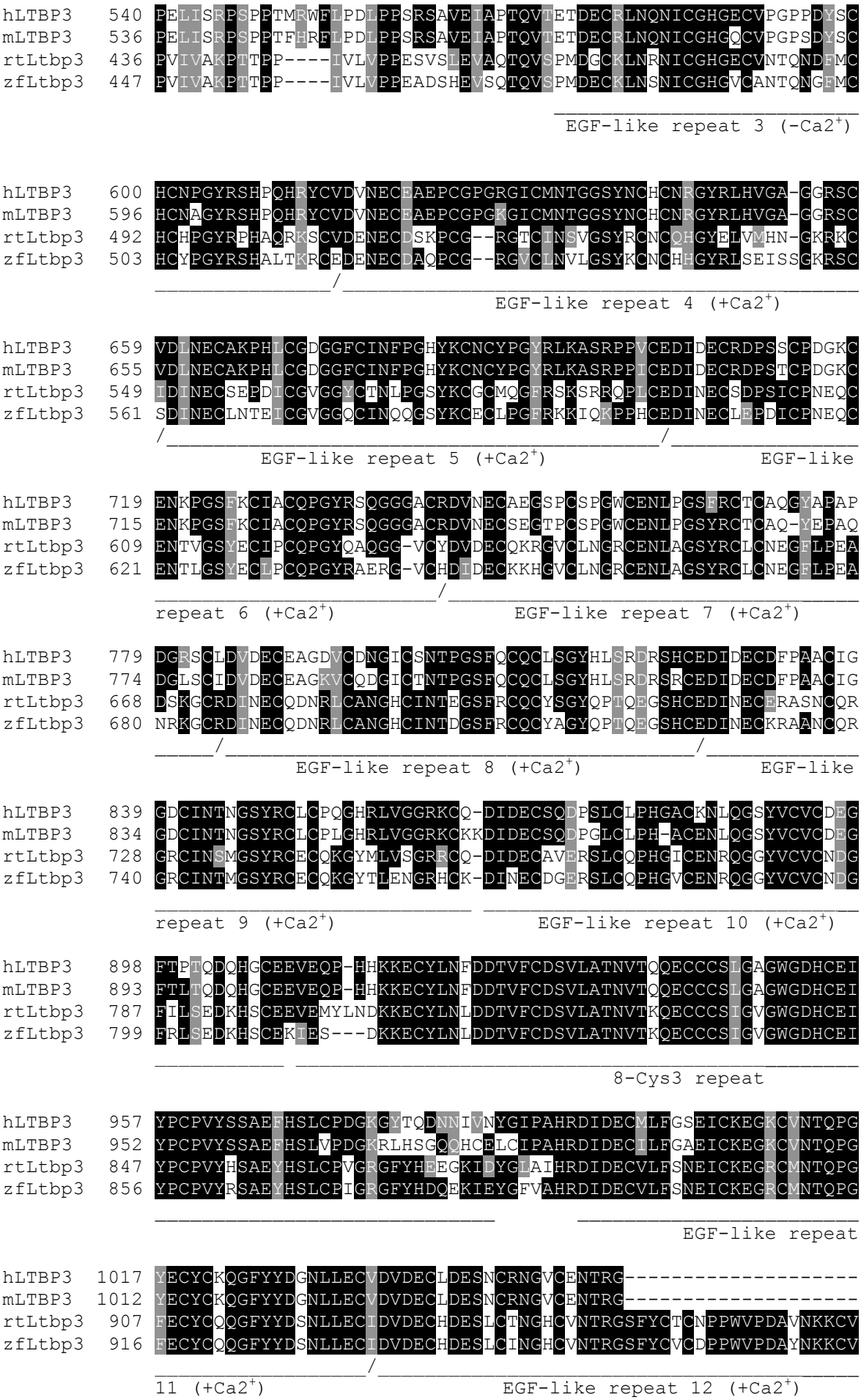
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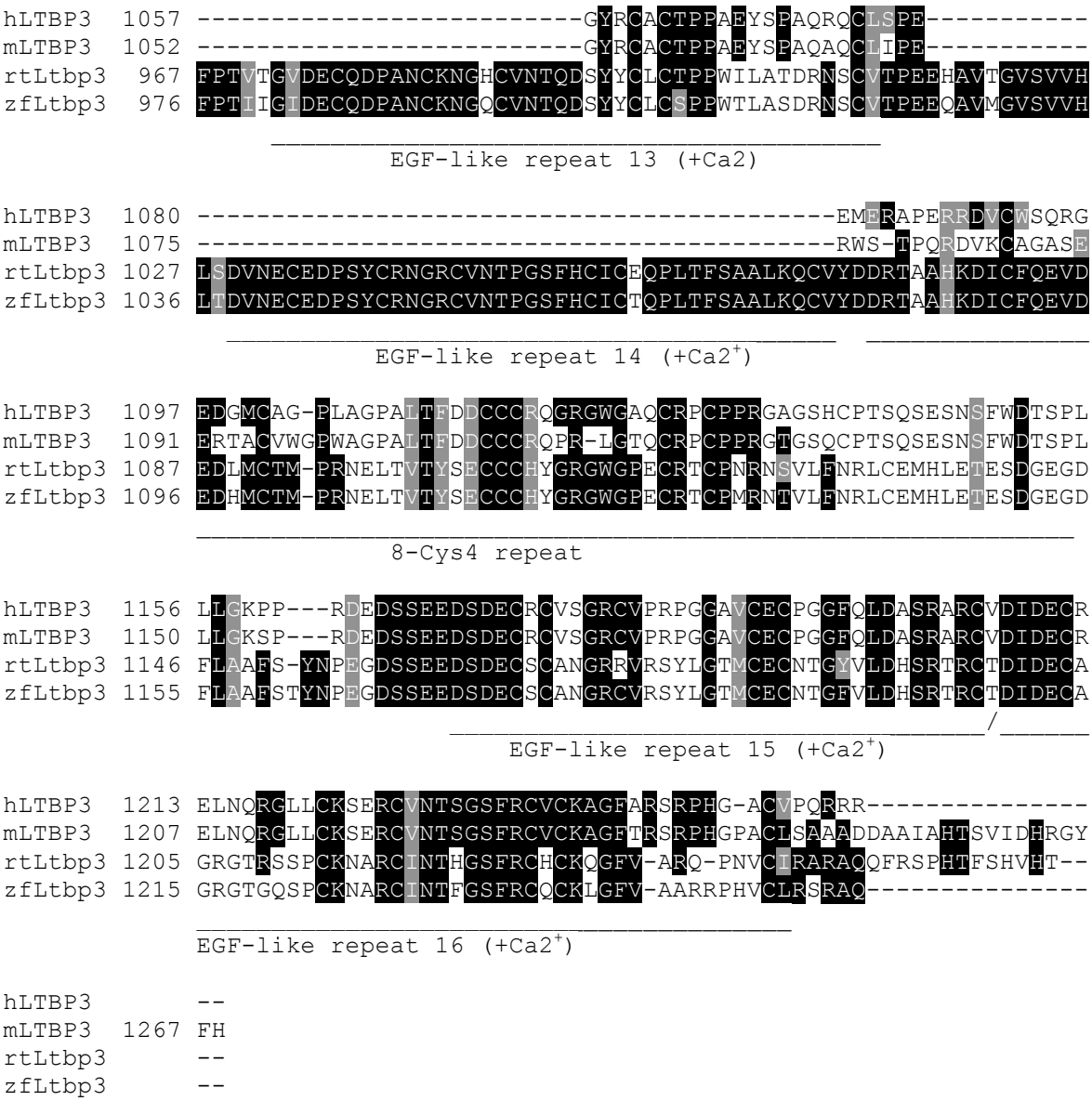
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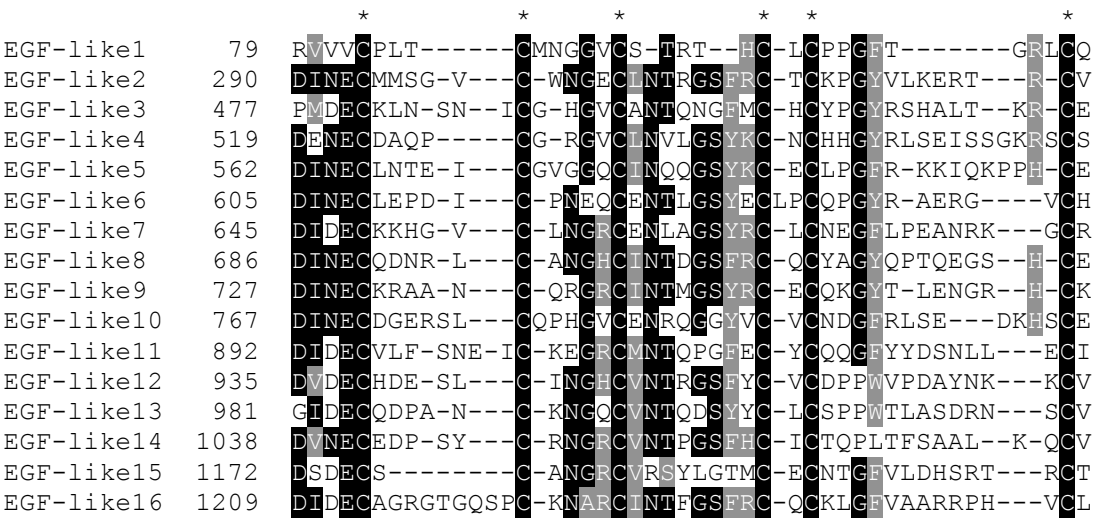
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EGF-like6 605 DINECLEPD-I---C-PNEQCENTLGSYECLPCQPGYR-AERG----VCH
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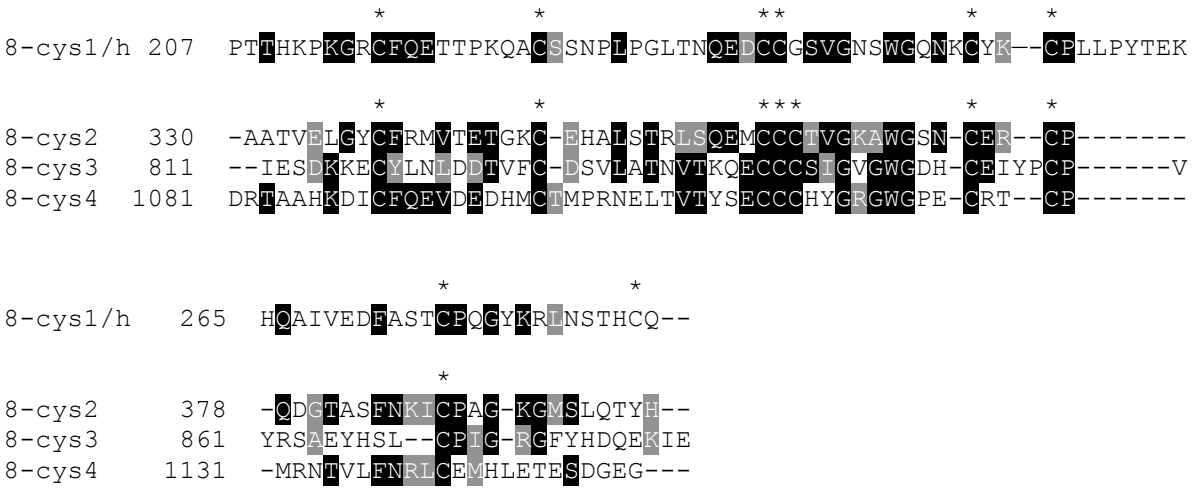
d.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8-cys3 811 --IESDKKECYLNLDDTVFC-DSVLATNVTKQECCCSIGVGWGDH-CEIYPCP------V
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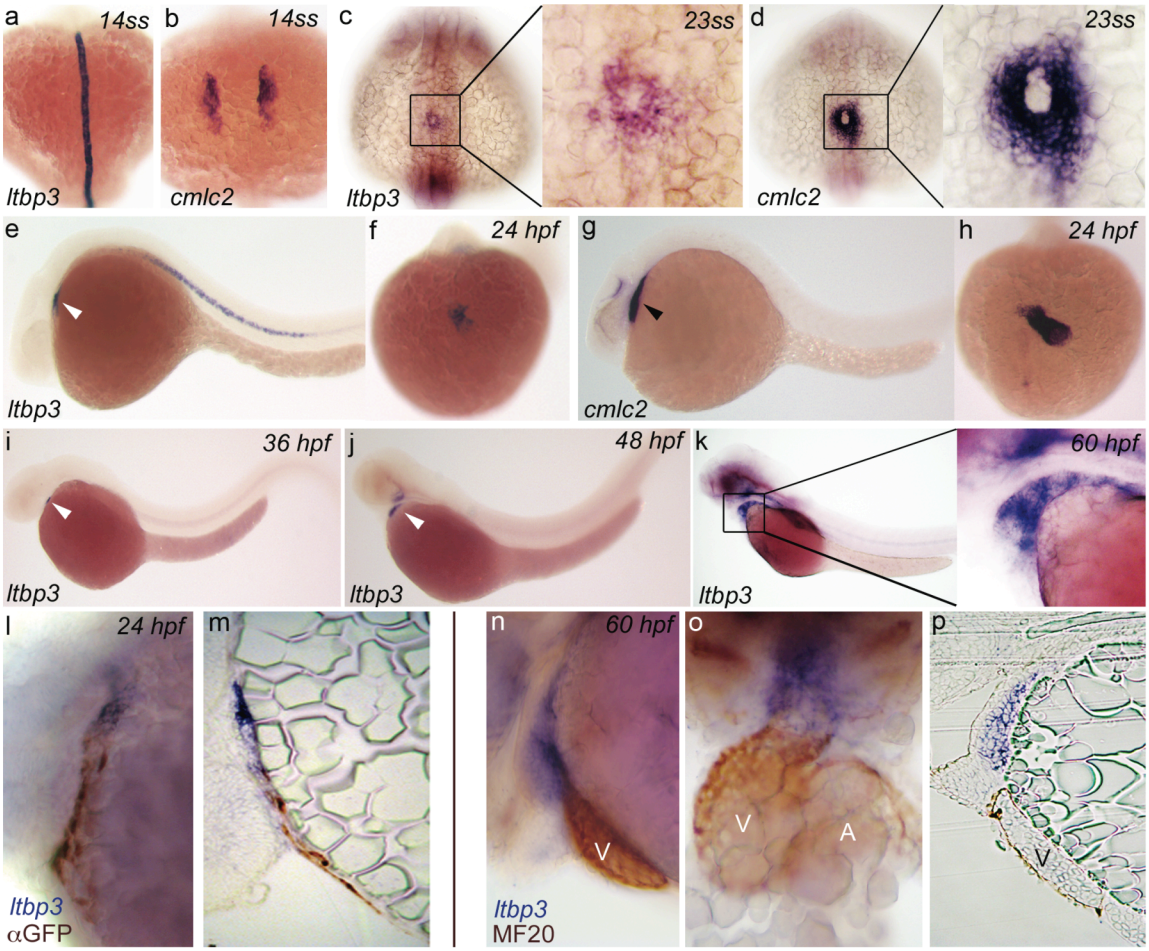
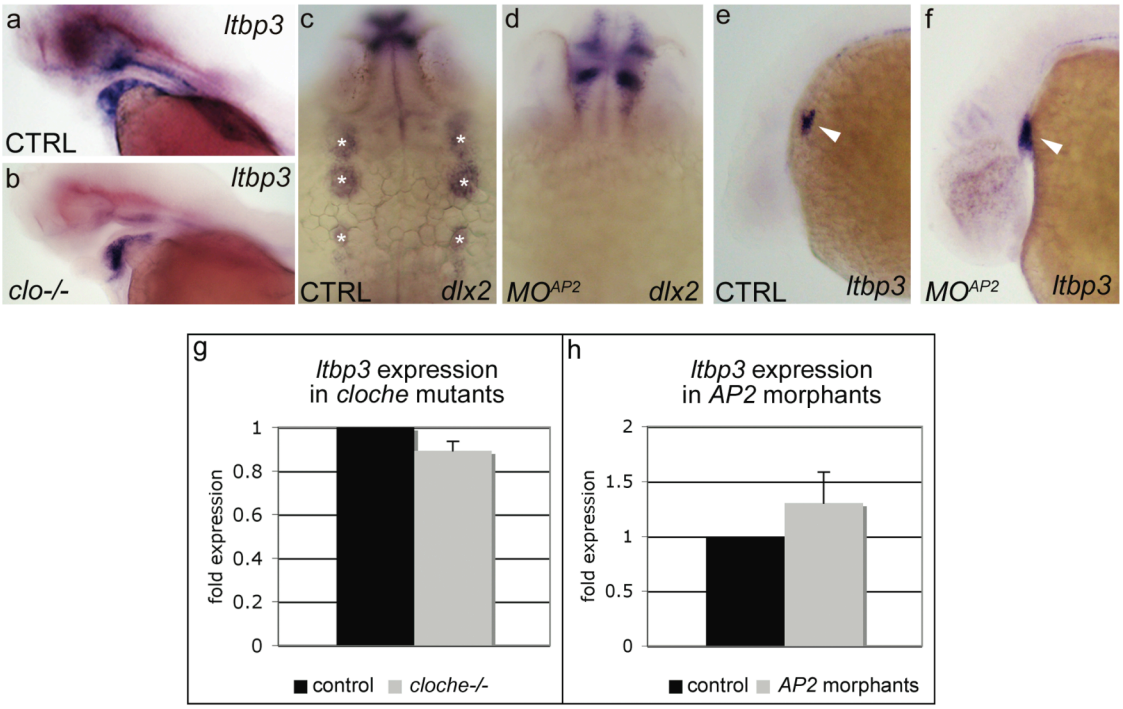
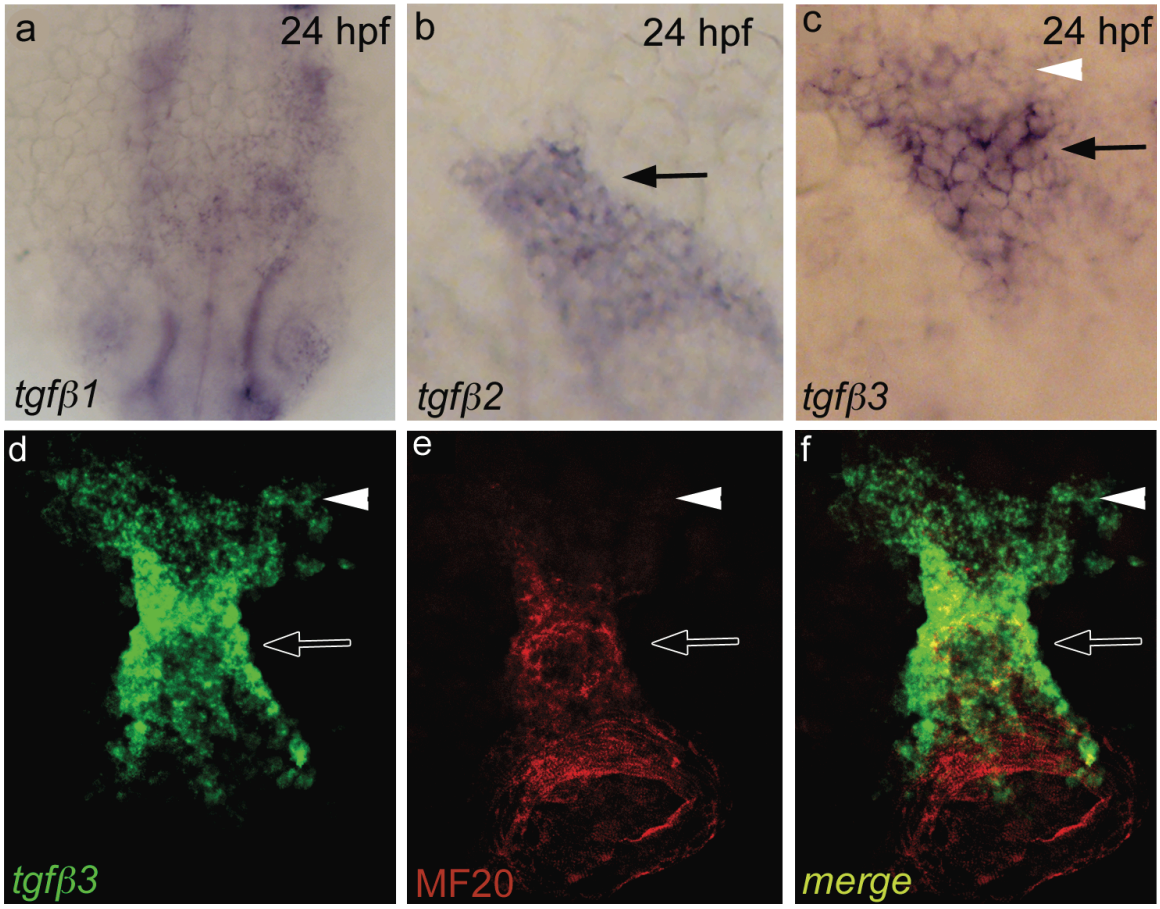


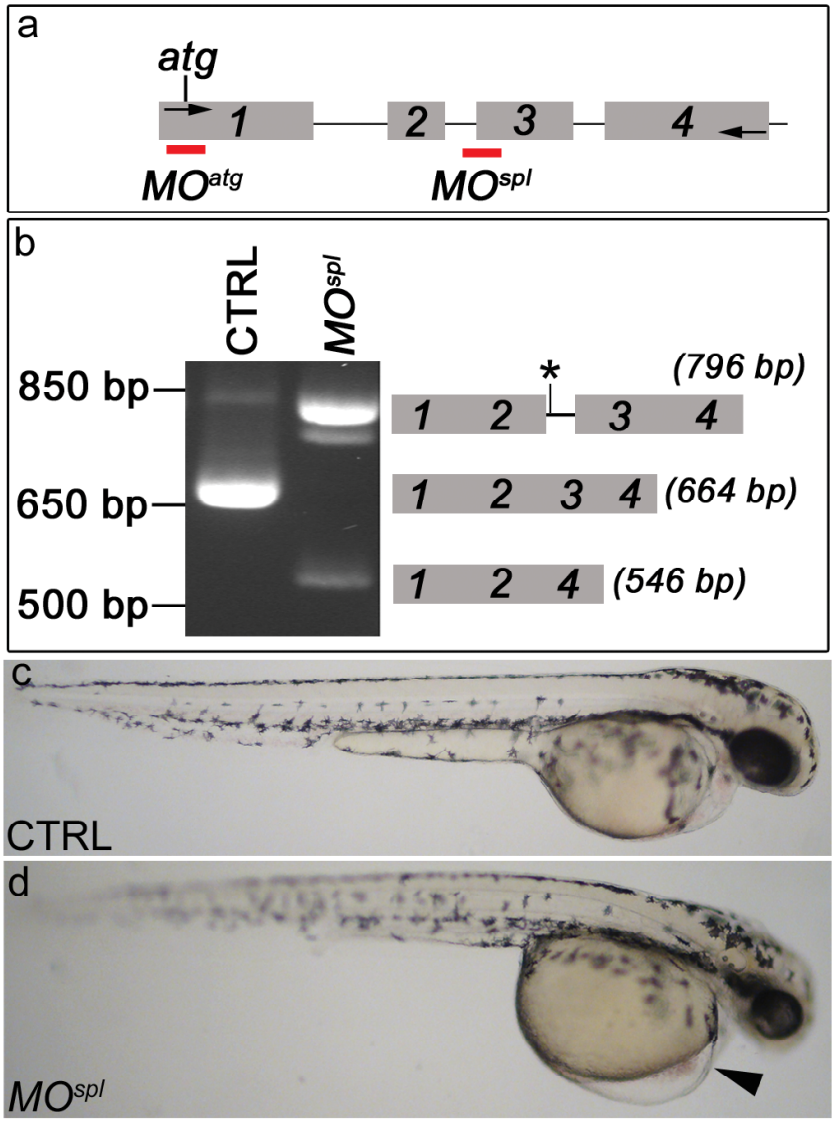
Supplementary Figure 2 – Developmental expression pattern of *ltbp3* transcripts in relation to myocardium. a–k, Embryos at the indicated developmental stages were processed for *in situ hybridization* with *ltbp3* or *cmlc2* riboprobes. *Ltbp3* transcripts are expressed initially in the notochord (**a**) but not in cardiomyocytes that differentiate in the anterior lateral plate mesoderm (**b**). *Ltbp3* transcripts appear subsequently in cells (**c**) that approximate the interior aspect of the cardiac cone (**d**). We speculate that *ltbp3* cells at this stage reside primarily in undifferentiated splanchnic mesoderm superior to *cmlc2*+ cells in this view. **e–k**, At subsequent developmental stages, *ltbp3* transcripts (white arrows) reside in close proximity to the outflow pole of the heart tube (black arrow in **g**) and maturing heart as the head rises dorsally. (**f**) and (**h**) are anterior views of the same embryos shown in (**e**) and (**g**). **l, m,** 24 hpf *Tg(cmlc2::GFP)* embryo co-stained with *ltbp3* riboprobe and an α-GFP antibody. **n-p**, 60 hpf embryo co-stained with *ltbp3* riboprobe and a muscle-specific antibody (MF20) that recognizes myocardial cells. Whole mounted embryos (**l**, **n**, **o**) and plastic sections (**m, p**) reveal that a majority of *ltbp3*+ cells (blue) are non-overlapping with myocardium (brown). ss=somite stage, hpf=hours post fertilization, V=ventricle A=atrium.



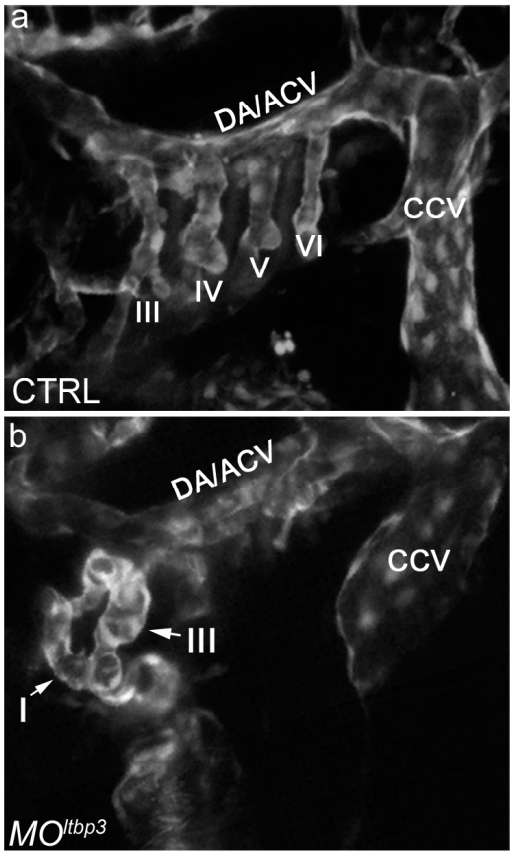
Supplementary Figure 3 – Analysis of *ltbp3* expression in embryos lacking endothelial and neural crest cells. a,b, 60 hpf *cloche* mutant embryos express *ltbp3* transcripts (n=9/10 for *clo-/-,* n=17/17 for siblings). **c–f,** 24 hpf embryos deficient in neural crest cells (MO^{AP2}) evidenced by the lack of *dlx2* staining (present in control, *) continue to express *ltbp3* transcripts (arrowheads; n=20/20 for all experimental groups). **g,h,** Quantitative PCR analyses were used to measure the relative abundances of *ltbp3* transcripts in *cloche* embryos at 60 hpf and MO^{AP2} morphants at 24 hpf compared to wild-type siblings and control embryos respectively. The threshold cycles (Cts) for *ltbp3* were normalized to those for β-actin for each experimental group in three independent experiments prior to calculating the averages and single standard deviations shown in the graphs. The apparent differences between experimental groups are not statistically significant.



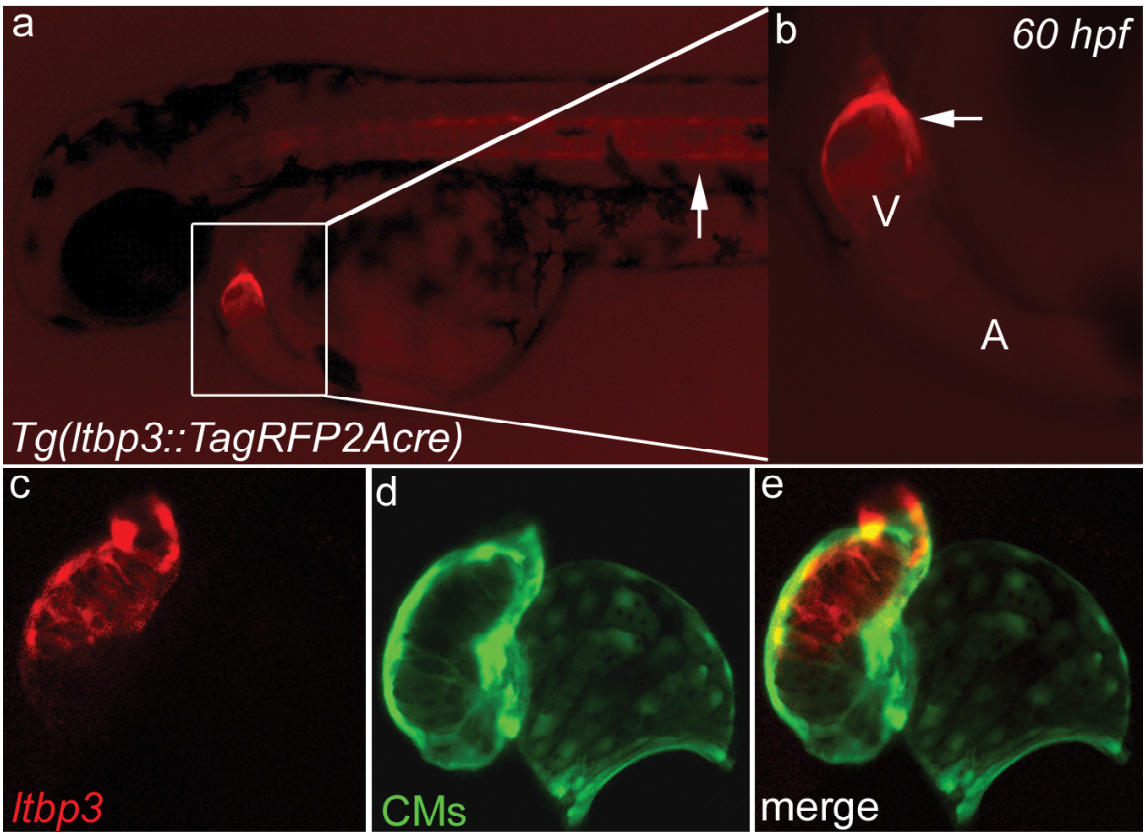
Supplementary Figure 4 – Expression analysis of *TGFβ* ligands in the zebrafish heart tube region. a–c, Zebrafish embryos at 24hpf were processed for *in situ hybridization* with riboprobes for *tgfβ1a*, *tgfβ2*, or *tgfβ3* and evaluated for ligand expression in the heart tube and extra-cardiac cells at its outflow pole. **a,** Anterior view of embryo demonstrating the absence of *tgfβ1a* transcripts in or near the heart tube (n=20/20). **b,** *tgfβ2* transcripts were observed in the heart tube (black arrow) but not in extra-cardiac cells at its outflow pole (n=>20/20 embryos). **c,** *tgfβ3* transcripts were present in what appeared to be a distal segment of the heart tube (black arrow) and in extra-cardiac cells at its outflow pole (white arrowhead; n=16/16). **d–f,** Double marker analysis with an antibody (MF20) that recognizes cardiomyocytes confirmed that *tgfβ3* transcripts overlap with a distal segment of the heart tube (black arrow) and approximate the extra-cardiac expression pattern of *ltbp3* transcripts (white arrowhead, compare1with Fig. 1 c–e, n=8/8).



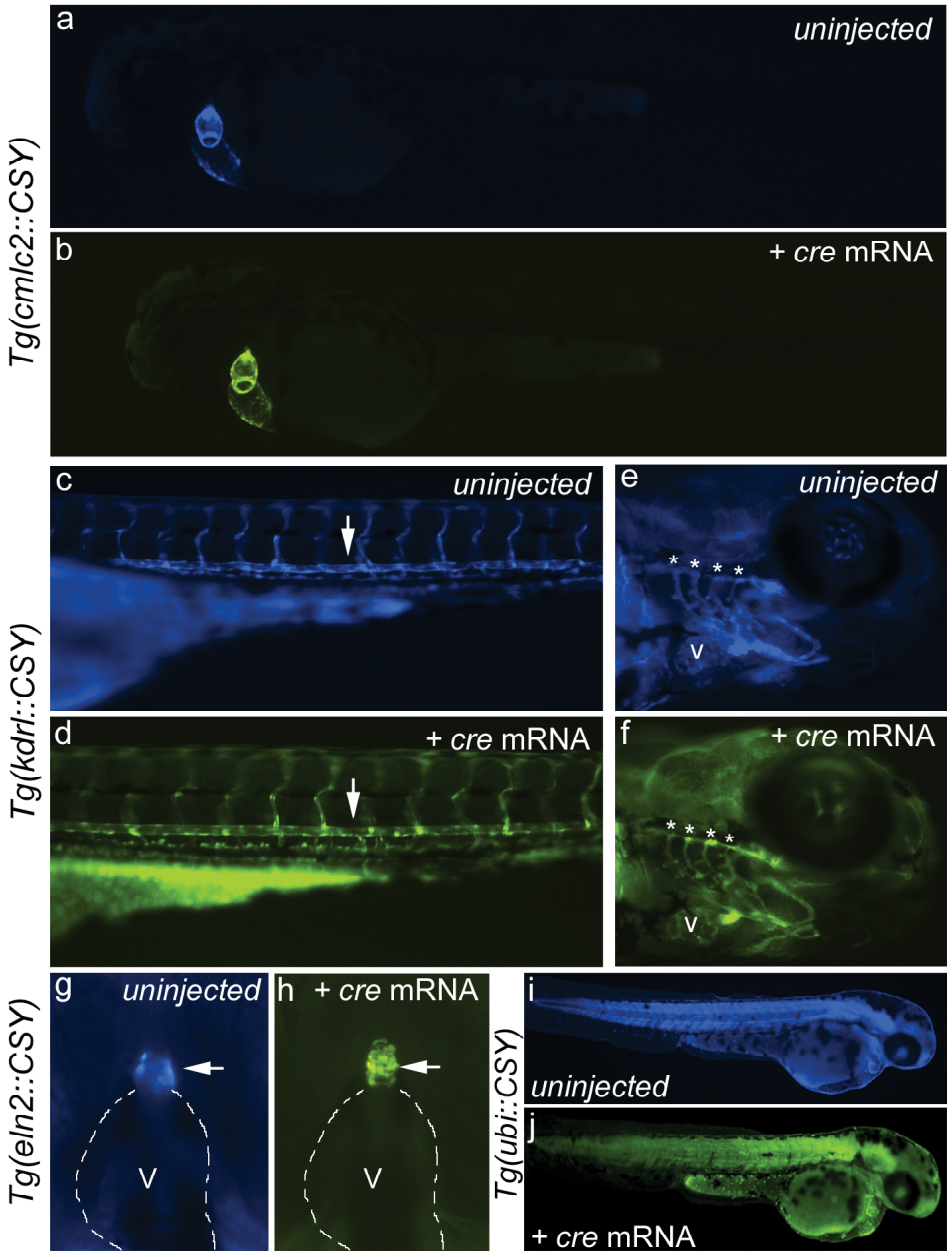
Supplementary Figure 5 – Validation of an anti-sense morpholino for knocking down Ltbp3. a, Schematic diagram of the first 4 *ltbp3* exons (not shown to scale) illustrating the locations of binding sites for two anti-sense *ltbp3* morpholinos, one targeting the initiation codon (MO^{atg}) and another targeting the splice acceptor site for intron 2 (MO^{spl}). The locations of binding sites for primers used in (**b**) are also shown (black arrows). **b,** RT-PCR reactions with primers shown in (**a**) and first strand cDNA templates from control and MO^{spl} embryos. Whereas control embryos express WT *ltbp3* transcripts, MO^{spl} embryos express improperly-spliced *ltbp3* mRNAs with frame-shift induced premature stop codons in intron 2 (*, top species) and in exon 5 (not shown; bottom species). **c, d,** Brightfield images of control and MO^{spl} embryos at 72 hpf. Grossly, MO^{spl} embryos display moderate pericardial edema (arrowhead) and mild body axis curvature defects that account for the head and tail being out of focus in this image.



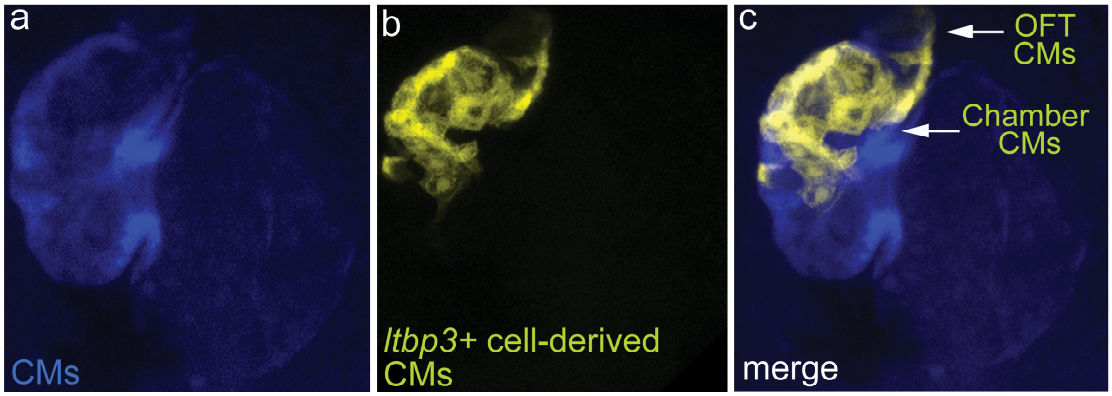
Supplementary Figure 6 – *ltbp3* morphants exhibit reductions in pharyngeal arch arteries. a,b, GFP+ pharyngeal arch artery (PAA) endothelium in 60 hpf *Tg(kdrl::GFP)* control and *MO*^{ltbp3} embryos. Whereas control embryos exhibit 4 PAAs (III-VI), morphant embryos display 1 PAA (III) (n=3/3 for WT; n=35/36 for *MO*^{ltbp3}). PAA I is out of view in the control embryo but visible in the morphant embryo because it fails to become remodeled in the morphant. DA=dorsal aorta; ACV=anterior cardinal vein; CCV=common cardinal vein.



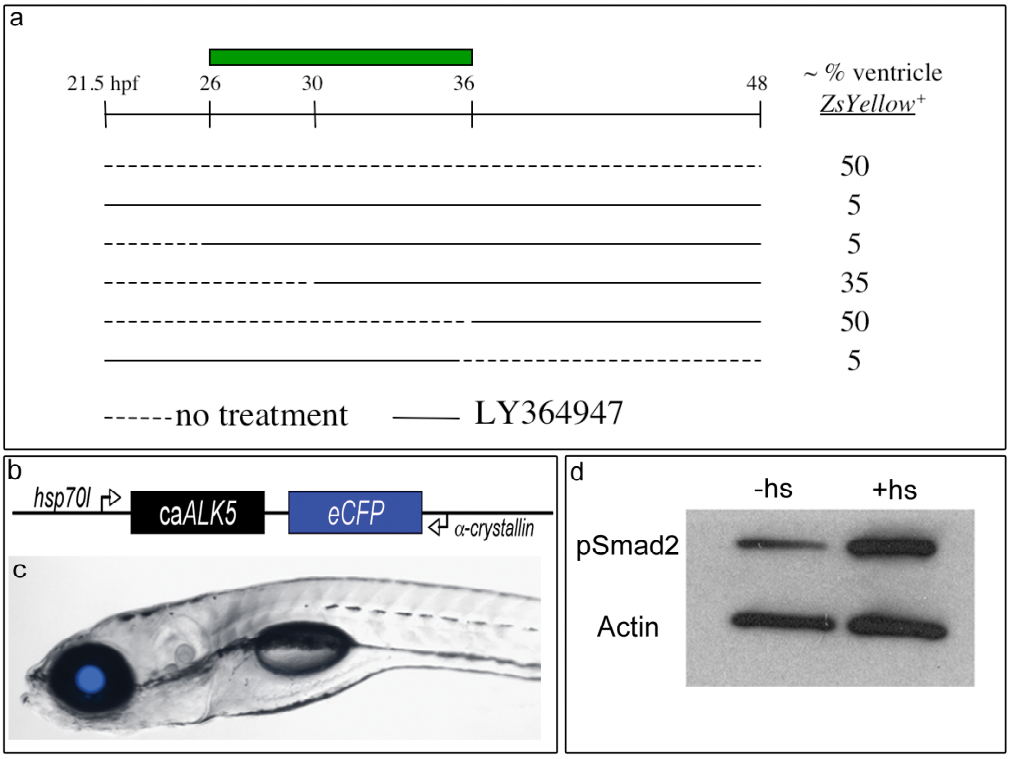
Supplementary Figure 7 – *Tg(ltbp3::TagRFP2Acre)* embryos express *TagRFP* protein in the notochord and heart. a, Confocal image of a *Tg(ltbp3::TagRFP2Acre)* embryo at 60hpf. A close up of the heart is shown in (**b**) *TagRFP* expression is seen in the notochord (arrow in **a**) and the distal ventricle (arrow in **b**). At this developmental stage, *ltbp3* transcripts are not expressed in the ventricle (Supplementary Fig. 2). However, due to the long half-life of *TagRFP*, the myocardial descendants of *ltbp3*+ cells in the distal ventricle continue expressing *TagRFP* protein. **c–e,** Confocal images of a 52hpf *Tg(ltbp3::TagRFP2Acre); Tg(cmlc2::GFP)* embryo demonstrating that *TagRFP* expression colocalizes with myocardial cells in approximately the distal half of the ventricle. V=ventricle, A=atrium CMs=cardiomyocytes.



Supplementary Figure 8 – Validation of Cre-responsive reporter strains. a–j, One-cell stage *Tg(cmlc2::CSY), Tg(kdrl::CSY), Tg(eln2::CSY),* and *Tg(ubi::CSY)* embryos were injected with *cre* recombinase mRNA and imaged at 52hpf (**b**), 72hpf (**d, f**), 5dpf (**h**), and 48hpf (**j**). Uninjected embryos (**a, c, e, g, i**) were processed in parallel. ZsYellow protein expression was never seen in uninjected embryos demonstrating that the reporter strains are not leaky. Injected embryos exhibited complete color switching. Arrows in (**c, d**) highlight the dorsal aorta. Asterisks (*) in (**e, f**) indicate where the four pharyngeal arch arteries meet the lateral dorsal aorta to highlight that all of the pharyngeal arch arteries express ZsYellow protein. Dorsal views of embryos are shown in (**g, h**), anterior up. Arrows highlight AmCyan (**g**) and Zsyellow (**h**) protein fluorescence in the outflow tract (e.g. the bulbus arteriosus). V=ventricle.



Supplementary Figure 9 – *ltbp3*+ cells give rise to OFT myocardium. a–c, Heart in *Tg(ltbp3::TagRFP2Acre); Tg(cmlc2::CSY)* embryo at 72 hpf demonstrating that cardiomyocytes (CMs) in the outflow tract (OFT) express ZsYellow protein.



Supplementary Figure 10 – TGFβ signaling is required between 26 and 36 hpf for zebrafish SHF development and validation of a *Tg(hsp70l::caALK5)* strain for inducible ubiquitous activation of TGFβ signaling. **a,** *Tg(ltbp3::TagRFP2Acre); Tg(cmlc2::CSY)* double transgenic embryos were exposed to a small molecule inhibitor of TGFβ signaling (LY364947) during the indicated developmental windows. At 72 hpf, we estimated the distal percentage of the total ventricle expressing ZsYellow protein in each embryo. The ZsYellow distribution was consistent between embryos in each experimental group (n>8/group). The green bar highlights the developmental window, 26-36 hpf, during which TGFβ function is required for accretion of myocardial cells to the ventricle from *ltbp3*+ cells. **b,** Schematic of the *Tg(hsp70l::caALK5)* transgene for heat-shock inducible ubiquitous activation of TGFβ signaling. The transgene is comprised of the zebrafish *hsp70l* promoter upstream of the coding sequence for a constitutively active TGFβ type I receptor (caALK5). This transgene includes a second cassette, comprised of the lens-specific α-crystallin promoter upstream of the coding sequence for enhanced cyan fluorescent protein (eCFP, Cerulean variant), for rapid visual identification of *Tg(hsp70l::caALK5)* embryos. **c,** 5 days post fertilization *Tg(hsp70l::caALK5)* embryo expressing eCFP in the lens. **d,** Western blot of protein extracts from *Tg(hsp70l::caALK5)* embryos heat shocked (+hs) or not (-hs) at 22 hpf and probed with α-pSmad2 and α-actin antibodies. In heat-shocked embryos, pSmad2 abundance normalized to Actin was 3.8-fold greater than in control embryos.

Supplementary (Full) Materials and Methods

Zebrafish husbandry and strains

Zebrafish were grown and maintained according to protocols approved by the Massachusetts General Hospital Institutional Animal Care and Use Committee.

Transgenic lines *Tg(cmlc2::dsRed2-nuc)*, *Tg(fli1::nEGFP)*, and *Tg(kdrl::GFP)* [formerly *Tg(flk1::GFP)*] were used to count cardiomyocytes, endocardial cells, and visualize pharyngeal arch arteries respectively [25-27]. Cell nuclei were counted in Z-stack confocal images using ImageJ software [28]. The *Tg(cmlc2::GFP)* transgenic strain and *cloche*^{m39} allele have been described previously [29, 30]. *clo*^{m39} embryos were identified by the absence of GFP fluorescence in the head and trunk in *Tg(kdrl::GFP); clo*^{m39} embryos.

Sequence analysis of zebrafish *ltbp3*

A partial cDNA predicted to encode a C-terminal fragment of zebrafish latent TGFβ binding protein 3 [image clone 4966012; accession numbers BI428840 and BI429284; pZL1*ltbp3*par(tial)] was obtained from a commercial source (Open Biosystems, Huntsville, AL) and sequenced on both strands. To acquire the full-length cDNA sequence, we performed 5' RACE with embryonic heart first-strand cDNAs and an *ltbp3*-specific primer, (5' - CCTGTTAGCCTCGGGCAGAAAG CCCTCATTACACAGACAT CGATAAGATCCTGCC – 3') according to manufacturer's protocols (Clontech, Mountain View, CA). An amplification product of approximately 2.5 Kb was cloned into pCRTOPOII (Invitrogen, Carlsbad, CA) and sequenced on both strands. Protein motifs were identified using InterProScan [33] and Motif Scan [34]. Protein alignments were constructed using ClustalW [35].

Morpholino injections

To generate *MO*^{AP2} embryos, one-cell stage embryos were injected with 1nl of anti-sense morpholinos targeting *tfAP2a* and *tfAP2c* at concentrations of 0.5ng/nl and 1ng/nl respectively [36]. To generate *ltbp3* morphants (*MO*^{ltbp3} embryos), one-cell stage embryos were injected with 1nl of anti-sense morpholinos targeting the initiation codon (5'-ATGCGAGACAATCAGCGAGGGCATG-3'; *MO*^{atg}; final concentration 3ng/nl) or the second-intron splice acceptor sequence (5'-ACCACctggacagatacatttattc -3'; *MO*^{spl}; final

concentration 4ng/nl; lower case letters indicate anti-sense intron sequence) of the *ltbp3* pre-mRNA. Embryos injected with MO^{atg} or MO^{spl} displayed identical phenotypes. The majority of MO^{ltbp3} embryos shown in the manuscript are MO^{spl} embryos. Uninjected control embryos were processed in parallel. Over the course of this study, we injected equivalent (and greater) quantities of several morpholinos targeting unrelated transcripts without eliciting the defects (individually or in combination) seen in *ltbp3* morphants. To determine the effectiveness of MO^{spl}-mediated *ltbp3* splicing inhibition, we generated first strand cDNAs from control and MO^{spl} embryos and performed quantitative PCR using standard reaction conditions and primers L35' (5' – GCCCTCGCTGATTGTCTCGCATCTGC – 3') and L33' (5' – GGGCGTGGTCTCCTGGAAGCACC – 3'). Furthermore, we queried the zebrafish genome (Zv9) using both morpholino sequences and did not retrieve genomic segments that differed by fewer than 8 nucleotides suggesting further that the *ltbp3* morphant phenotype is not confounded by off-target effects.

Single riboprobe whole mount *in situ hybridization*

Single riboprobe whole mount *in situ hybridizations* were performed in eppendorf tubes essentially as described [37] using digoxygenin-labeled anti-sense RNA probes to *ltbp3*, *cmlc2*, *dlx2*, and *tie1* [36, 38, 39]. The *ltbp3* riboprobe was transcribed with SP6 polymerase from pZL1*ltbp3*par digested with EcoRI. Complementary DNAs encoding zebrafish Tgfβ1a (accession number BC162361) and Tgfβ3 (accession number, BC081579) were obtained from a commercial source (Open Biosystems, Huntsville, AL), subcloned into pKSII+, and riboprobes were generated with T3 polymerase after linearization of the template with NotI enzyme. The zebrafish *tgfβ2* coding sequence (accession number NM_194385) was amplified from wild-type cDNA, subcloned into pCRBluntTOPOII, and the riboprobe was generated using SP6 polymerase after linearization with NotI enzyme. Probes were synthesized using a DIG RNA Labeling Kit (SP6/T7; Roche Applied Science, Mannheim, Germany). Nitro-Blue Tetrazolium Chloride/5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine (NBT/BCIP; Roche Applied Science, Mannheim, Germany) were used as chromogenic substrates.

Non-fluorescent dual *in situ hybridization* and immunohistochemistry

Twenty-four hours post-fertilization (hpf) *Tg(cmlc2::mGFP)* [29] or 60 hpf WT embryos were processed for *in situ hybridization* and immunohistochemistry using a modified

protocol for single riboprobe whole-mount *in situ hybridization* [37]. Unless otherwise specified, all steps were performed at RT. During incubation of embryos with the α-DIG antibody, mouse α-GFP [for 24 hpf embryos; GFP (B-2); Santa Cruz Biotechnologies, Santa Cruz, CA] or striated muscle-specific MF20 [40] antibodies were included at dilutions of 1:50. After NBT/BCIP staining, embryos were washed 2X for 15 min. with PBT (PBS + 0.1%Tween), 3X for 15 min. with PBX (PBS + 0.3% Triton X-100), and blocked in PBX with 2% BSA, 5% goat serum, and 1% DMSO for 60 min. Embryos were incubated overnight at 4°C with an α-mouse HRP-linked secondary antibody (Cellular Signaling Technologies, Danvers, MA) diluted 1:200 in block solution. Embryos were washed 4X for 30 min. in PBX. Antibody signals were developed with diaminobenzidene (DAB) substrate. Embryos were incubated with DAB solution [0.5 mg/ml Diaminobenzidene (Sigma Aldrich, St. Louis, MO) in PBT + 1% DMSO] for 30 minutes. The DAB solution was replaced with fresh DAB solution containing 0.0006% H_20_2 and the incubation was continued for 5 minutes. The reaction was stopped by washing twice in PBX. Embryos were stored in 4% PFA at 4°C. For sectioning, embryos were embedded in JB-4 plastic resin (Polysciences Inc., Warrington, PA) and 15 mm sections were cut using a Leica microtome (Leica Microsystems GmbH Wetzlar, Germany).

Fluorescent dual *in situ hybridization* and immunohistochemistry

Embryo fixation, proteinase K treatment, prehybridization, and hybridization were performed essentially as described [41] with minor modifications. Prehybridization for 3 hours, hybridization overnight (600ng probe/200ml HYB+;[41]), and SSC washes were performed at 60°C. All other washes were performed at RT. Following hybridization, embryos were washed for 15 min. each with 3:1, 1:1, and 1:3 of {HYB-:2X SSC; [41]}, 2X for 15 min. with 2X SSC, 2X for 30 min. with 0.2X SSC and 2X for 5 min. at RT with PBS. Embryos were blocked for 60 min. at RT in MNB solution [150 mM maleic acid, 100 mM NaCl, pH=7.5 plus 2% Blocking Reagent (Roche Applied Science, Mannhein, Germany)] and incubated overnight at 4°C with α-digoxigenin POD (Roche Applied Science, Mannheim, Germany) and MF20 [40] antibodies diluted 1:500 and 1:50 respectively in MNB solution. Embryos were washed 4X for 20 min. in MN (no blocking reagent) solution and 2X for 5 min. in PBS. Embryos were incubated for 60 min. at RT in TSA Plus Fluorescein-tyramide working solution (Perkin Elmer, Waltham, MA) and washed 2X for 15 min. with PBT (PBS+0.1% Tween). Embryos were incubated overnight at 4°C with Alexa Fluor 546 goat α-mouse IgG (Invitrogen, Carlsbad, CA) diluted 1:200

in MNB solution. Embryos were washed 5X for 10 min. with PBST prior to storage in PBS at 4°C.

TSA double fluorescent *in situ hybridization*

Tyramide signal amplification (TSA) double fluorescent *in situ hybridizations* were performed as described [41]. Flourescein-labeled *nkx2.5* riboprobe [42] was synthesized using the DIG RNA Labeling Kit (SP6/T7) and Fluorescein RNA Labeling Mix (Roche Applied Science, Mannheim, Germany). Prehybridization, hybridization (600ng probe/200ml HYB+;[41]), and washes were performed at 65°C. TSA Plus Fluorescein tyramide and Cyanine 5 tyramide working solutions (Perkin Elmer, Waltham, MA) were used to develop the *nkx2.5* and *ltbp3* riboprobe signals respectively.

Immunohistochemistry

Unless otherwise stated, manipulations were performed at RT. Prior to fixation, embryos were treated with 0.5M KCl to stop the hearts. Embryos were fixed at 4°C in 80% MeOH/20% DMSO for at least four hours at 4°C, dehydrated in a methanol series, and stored in 100% MeOH at –20°C for at least 2 days. Embryos were re-hydrated to 1XPBS again in a methanol series and blocked with 1XPBS, 1%BSA, 1%Triton-X100, 0.1% DMSO for 4 hours. Embryos were incubated with primary antibodies diluted in block solution overnight at 4°C. Embryos were washed 1X in PBS for 5 min. and 1X in PB0.5X (PBS + 0.5% TritonX-100) for 45 min. Embryos were incubated with secondary antibodies diluted in PB0.5X for two hours. Embryos were rinsed in TST (20mM Tris pH=7.6, 150 mM Sodium Chloride, and 0.5% Triton X-100) for 45 minutes prior to storage at 4°C in TST. Primary antibodies specific for Tropoelastin2 (Eln2) [43], atrial myosin heavy chain (S46; [40]), and myosin heavy chain (MF20; [40]) were used at dilutions of 1:1000, 1:50, 1:50 respectively. The following secondary antibodies were used at dilutions of 1:200. For detecting Eln2 we used Alexa Fluor 488 goat α-rabbit IgG (Invitrogen Corp. Carlsbad, CA). For detecting atrial myosin heavy chain and myosin heavy chain, we used FITC-conjugated rat α-mouse IgG1 (Caltag Laboratories, Burlingame, CA) and rhodamine-conjugated goat α-mouse IgG2 (Santa Cruz Biotechnology; Santa Cruz, CA) respectively.

pSmad2 immunohistochemistry

Unless otherwise stated, all steps were performed at RT. Embryos at 24 hpf were fixed in MEMFA {0.1 M MOPS, pH 7.4, 2 mM EGTA, pH 8, 1 mM MgSO4, 3.7% formaldehyde; [44]} + 5mM Iodacetate for 2 hours, washed 1X in 50% MeOH/MEMFA for 5 min., washed 1X in 100% MeOH for 5 min., and stored in 100% MeOH at –20^{o}C overnight. Embryos were rehydrated through 5, 5-min. washes with increasing percentages of PBS in MeOH: 30%, 50%, 70%, 90%, and 100%. Embryos were washed 2X for 5 min. in PBBT (PBS plus 2mg/ml BSA+0.1%Triton), and washed 1X for 15' in PBBT. Embryos were blocked in PBBT + 10% goat serum for 60 min. Embryos were incubated overnight at 4^{o}C on a NUTATOR (TCS Scientific Corp., New Hope, PA) with primary antibodies, rabbit pSmad2 (Ser465/467) antibody (#3101; Cell Technologies, Danvers, MA) and mouse MF20 antibody [40], both diluted 1:50 in block solution. Embryos were subsequently washed with PBBT 5X over the course of 2 hours. Embryos were incubated overnight at 4^{o}C on a NUTATOR with secondary antibodies, α-rabbit Alexa Fluor 488 IgG (Invitrogen, Carlsbad, CA) and α-mouse Alexa Fluor 546 IgG (Invitrogen, Carlsbad, CA) both diluted 1:200 in block solution. Embryos were washed again with PBBT 5X over the course of 2 hours. Embryos were stored in PBS at 4^{o}C prior to imaging.

Construction of ATI and ITK cassettes for flanking BAC inserts with *Tol2* and I-SceI sequences.

Two selectable cassettes were generated for flanking bacterial artificial chromosome (BAC) inserts, through BAC recombineering, with I-SceI meganuclease sites and *Tol2* elements both known to increase the germ-line transmission frequency of transgenes [45-47]. The ATI cassette contains the ampicillin resistance gene (*AmpR*) upstream of the 5'/left *Tol2* arm [48] upstream of an I-SceI site. The ITK cassette contains an I-SceI site upstream of the 3'/right *Tol2* arm [48] upstream of the kanamycin resistance gene (KanR). Further details and sequences available upon request.

Isolation of the *Tg(nkx2.5::ZsYellow)* transgenic strain

A BAC (DKEY-9I15) with ~36Kb and ~194Kb upstream and downstream respectively of the first *nkx2.5* exon was obtained. λ–red-mediated BAC recombineering [49-51] was used to replace the first exon coding sequence with the ZsYellow coding sequence, to flank the BAC insert with ATI and ITK cassettes, and to trim the downstream genomic

region to ~20Kb. For germ-line transmission, the BAC insert was released by restriction digest, EtOH precipitated, and injected into the cell of one-cell stage embryos. Candidate founders were selected based on strong transient fluorescence, grown to sexual maturity, and screened for germ-line transmission in test crosses.

Construction of expression and targeting cassettes *TagRFP2AcreFKF* and *loxPAmCyanSTOPloxPZsYellowFKF*

Two expression cassettes for Cre/Lox-mediated lineage tracing were generated using standard molecular cloning methods and/or Gateway technology (Invitrogen, Carlsbad, CA). The expression cassette, *TagRFP2Acre*, for co-expressing a red fluorescent protein, TagRFP*,* (Evrogen, Moscow, Russia) and Cre recombinase was generated by separating the open reading frame sequences with the viral *2A* sequence [52].

A Cre-responsive "color switching" cassette, *loxPAmCyanSTOPloxPZsYellow (AmCyan-Switch-Yellow;CSY)*, for irreversibly marking cells expressing Cre recombinase was generated using Gateway technology. A Gateway middle clone (pME*loxPAmCyanSTOPloxP*) was generated that contains a floxed *AmCyan-STOP* sequence for expression of the AmCyan protein (Clontech, Mountain View, CA) without transcriptional readthrough [53]. A 3' entry clone (p3E-*ZsYellow*) containing the coding sequence for ZsYellow protein (Clontech, Mountain View, CA) was also generated. pME*loxPAmCyanSTOPloxP,* p3E-*ZsYellow,* and a 5' entry clone (p5E-*cmlc2*) containing 0.85Kb of the *cardiac myosin light chain* promoter [54] were combined in an LR reaction with destination vector pDestTol2pA2 [55] to generate *pDestTol2pA2cmlc2::loxPAmCyanSTOPloxPZsYellow*. A similar LR reaction in which p5E-*cmlc2* was replaced by a 5' entry clone (pENTR5*'_ubi*) containing 3.5Kb of the zebrafish ubiquitin promoter [56] was performed to generate *pDestTol2A2ubi::loxPAmCyanSTOPloxPZsYellow*. For BAC recombineering, both *TagRFP2Acre* and *loxPAmCyanSTOPloxPZsYellow* sequences were cloned upstream of the *FLP*-recombinase excisable selectable marker, *Frt-KanR-Frt* (FKF) [49] to generate plasmids containing the *TagRFP2AcreFKF* and *loxPAmCyanSTOPloxPZsYellowFKF* targeting cassettes. Further details and sequences available upon request.

Isolation of the *Tg(ltbp3::TagRFP2Acre)* transgenic driver strain

Pools of BAC clones from the Zebrafish BAC DanioKey library (Imagenes, Berlin, Germany, formerly RZPD) were screened by PCR to identify a BAC containing the first *ltbp3* exon. This strategy identified a BAC with ~ 25Kb and ~40Kb of genomic sequence upstream and downstream respectively of the first *ltbp3* exon. Using BAC recombineering, we replaced the coding sequence in the first *ltbp3* exon with *TagRFP2AcreFKF.* Again using BAC recombineering, we flanked the entire *ltbp3::TagRFP2Acre*-containing BAC insert with the ATI and ITK cassettes to facilitate germ-line transmission. WT embryos were injected with the BAC and *Tol2* transposase mRNA. Candidate founders were selected based on strong transient fluorescence, grown to sexual maturity, and screened for germ-line transmission in test crosses.

Isolation of *Tg(cmlc2::CSY)*, *Tg(kdrl::CSY)*, *Tg(eln2::CSY)*, and *Tg(ubi::CSY)* reporter strains

Methods for generating the myocardial *cmlc2::CSY* and ubiquitous *ubi::CSY* transgenes are presented above. To generate the endothelial reporter transgene, we obtained a BAC (CH211-276G21) with ~50 and ~120Kb of genomic sequence upstream and downstream respectively of the first exon for the endothelial marker, *kdrl* (formerly termed *flk1*) [27, 57]. BAC recombineering was used to replace the coding sequence in the first exon with *loxPAmCyanSTOPloxPZsYellow,* to flank the BAC insert with ATI and ITK cassettes, and trim the BAC to contain only ~20Kb upstream and downstream of *loxPAmCyanSTOPloxPZsYellow*.

To generate the smooth muscle precursor cell reporter transgene, we obtained a BAC (DKEY-83D6) with ~184Kb and ~32Kb upstream and downstream respectively of the first exon for *eln2*. BAC recombineering was used to replace the coding sequence in the first exon with *loxPAmCyanSTOPloxPZsYellow*, to flank the insert with *ATI* and *ITK* cassettes, and to trim the upstream genomic region to ~40Kb.

All four reporter transgenes were co-injected with *Tol2* transposase mRNA into WT embryos. Candidate founders were selected based on strong transient fluorescence, grown to sexual maturity, and screened for germ-line transmission in test crosses. Cre-

dependent AmCyan to ZsYellow color switching was confirmed by injecting F1 embryos with mRNA encoding Cre recombinase.

Isolation of the *Tg(hsp70::caALK5)* transgenic strain, western blotting, and Rescue of *MO^{ltbp3}* embryos

Gateway technology (Invitrogen, Carlsbad, CA) was used to generate a transgene for heat-shock inducible ubiquitous activation of *TGFβ* signaling. First, a middle clone (pME*ALK5T204D-HA*) was generated containing the coding sequence for a constitutively active HA-tagged isoform of the human *TGFβ* Type I ALK5 receptor (caALK5) [58]. pME*ALK5T204D-HA* was combined with p5E-*hsp70l*, p3E-polyA [55] and the destination vector pDestTol2pA2AC in an LR reaction to generate pDestTol2pA2AC*hsp70l::ALK5T204D-HApolyA* [55]. This destination vector is a modification of pDestTol2pA2 to include a transgene [*Tg(α-crystallin::eCFP)*;[59]] for rapid identification of embryos and adults carrying the transgene by virtue of lens fluorescence. The pDestTol2pA2AC*hsp70l::ALK5T204D-HApolyA* plasmid was injected with *Tol2* transposase mRNA into WT embryos. Candidate founders were selected based on strong transient lens fluorescence, grown to sexual maturity, and screened for germ-line transmission in test crosses.

Twenty-two hpf *Tg(hsp70::caALK5)* embryos were heat shocked at 37^{o}C for 60 min. Control embryos carrying the same transgene, but not heat shocked, were processed in parallel. At 24 hpf, 20 embryos/group were deyolked in Ringer's solution [60], homogenized with a microfuge pestle in 50μl SDS Sample Buffer [60], heated to 95^{o}C for 5 min., and spun on high in a microfuge at R.T. for 2 min. One embryo equivalent of protein from each experimental group was separated on a 12% SDS-PAGE gel before being transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with 5% milk in TBS and probed with 1:1000 dilutions of a rabbit pSmad2 (Ser465/467) antibody (#3101; Cell Technologies, Danvers, MA) and goat α-actin (C-11) antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). After being washed, the membrane was subsequently probed with HRP-linked α-rabbit (Cellular Signaling Technologies, Danvers, MA) and α-goat secondary antibodies (Santa Cruz Biotechnologies, Santa Cruz, CA) at dilutions of 1:1000 prior to signal detection with an electrochemiluminescence reagent. Quantification of *TGFβ* activation was performed using ImageJ software [28].

Tg(ltbp3::TagRFP2Acre); Tg(cmlc2::CSY); Tg(hsp70l::caALK5) triple transgenic embryos were injected with MO^{ltbp3}. At 22 hpf, half of the injected embryos were heat shocked for 60 min. at 37°C. At 72 hpf, embryos were fixed, inspected visually for ventricular ZsYellow expression, and subsequently binned into one of three categories: 1) embryos with ZsYellow expression in approximately the distal half (~50%) of the ventricle; 2) embryos with ZsYellow expression in approximately the distal one quarter (~25%) of the ventricle; and 3) embryos with *ZsYellow* expression in approximately the distal one twentieth (~5%) of the ventricle. Uninjected triple transgenic embryos, not heat shocked were processed in parallel.

Dye labeling

Cell Tracker Red dye was obtained from a commercial source (Invitrogen, Carlsbad, CA) and diluted to 10mM in DMSO. The stock was diluted with E3 to a working concentration of 10μM. Twenty-four hpf *Tg(nkx2.5::ZsYellow); Tg(cmlc2::CSY)* double transgenic embryos were injected with a single or multiple 1nl drop(s) of dye in the $ZsYellow^{+}$, $AmCyan^{-}$ region contiguous to the heart tube outflow pole. Photographs of embryos were taken immediately following injection and again at 48 and/or 72 hpf. It is difficult to obtain high-resolution images of the heart tube at 24 hpf in live embryos because the head obscures the heart tube.

BrdU staining

BrdU incorporation and staining were performed essentially as described [61]. Twenty-seven hpf MO^{ltbp3} and control *Tg(nkx2.5::ZsYellow); Tg(cmlc2::CSY)* double transgenic embryos in E3 were chilled on ice for 15 minutes, transferred to a pre-chilled 10mMBrdU/15%DMSO solution and incubated on ice for 20 minutes. Embryos were transferred to E3 pre-warmed to 28.5°C and incubated for 15 minutes at 28.5°C prior to fixation. The methanol dehydration/rehydration steps were omitted because they cause the endogenous GFP signal to disappear. Embryos were digested with 10mg/ml Proteinase K for 10 min. at RT. Mouse α-BrdU (Roche Applied Science, Mannheim, Germany) and α-mouse Alexa Fluor 546 (Invitrogen, Carlsbad, CA) primary and secondary antibodies were used at dilutions of 1:100 and 1:200 respectively. Following antibody staining and washes, embryos were incubated with 0.5μg/ml 4',6-diamidino-2-phenylindole (DAPI) in 1XPBS for 15 min in the dark at RT. After three 1XPBS washes, the embryos were mounted and imaged.

DAPI+ and DAPI+/BrdU cells were quantified in the ZsYellow+ AmCyan- region of at the heart tube outflow pole. The average numbers of cells (DAPI+) in this region were 59 and 57 for WT and morphant embryos respectively. The average numbers of BrdU+ cells in this region were 5.5 and 0.3 for WT and morphant embryos respectively. We report BrdU+ cells as a percentage of total cells in the ZsYellow+ AmCyan- region to ensure that the observed decrease in proliferation wasn't an artifact of fewer cells being analyzed.

Small molecule treatment

LY364947 was obtained from a commercial source (Tocris Bioscience, Ellisville, MO) and dissolved in DMSO at a final concentration of 5mM. Embryos were exposed to LY364947 by immersing them in E3 containing the small molecule at the indicated concentrations. To evaluate chemically treated embryos for the presence or absence of pSmad2 epitopes, we exposed wild-type embryos to 60μM LY364947 between 18 and 24 hpf prior to fixation at 24 hpf. To evaluate chemically treated embryos for reductions in *ltbp3*$^{+}$ cell-derived myocardium, we exposed *Tg(ltbp3::TagRFP2Acre); Tg(cmlc2::CSY)* double transgenic embryos to 60μM LY364947 between 24 and 48 hpf prior to analysis at 72 hpf. To evaluate chemically treated embryos for the presence or absence of Eln2 epitopes, we exposed wild-type embryos to 60μM LY364947 between 24 and 48 hpf prior to fixation at 60 hpf. To determine the developmental stages during which TGFβ signaling is required for the formation of distal myocardium, *Tg(ltbp3::TagRFP2Acre); Tg(cmlc2::CSY)* double transgenic embryos were treated during the indicated developmental windows with 60μM LY364947. At 72hpf, we estimated the distal percentage of the total ventricle expressing ZsYellow for each embryo.

Microscopy

Live or whole mounted embryos and histological sections were imaged on a Nikon 80i compound microscope (Nikon Instruments Inc, Melville, NY) with Retiga 2000R high speed CCD camera (QImaing, Surrey, BC, Canada) and NIS-Elements advanced research image acquisition and analysis system (Nikon Instruments Inc, Melville, NY). Confocal microscopy was performed using a Zeiss LSM5 Pascal Laser Scanning

Microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY). AmCyan and eCFP/Cerulean fluorescent proteins were excited with a 405 nm blue diode laser and imaged through a 475nm long pass filter. GFP was excited with a 488nm Argon laser and imaged through a 505-536nm filter. ZsYellow protein was excited with a 514nm Argon laser and imaged through a 530nm long pass filter. TagRFP was excited with a 543nm HeNe laser and imaged through a 560nm long pass filter. In some instances, hearts were dissected from fixed embryos prior to imaging. In other instances, the head was removed from the fixed embryos to gain an unimpeded view of the heart *in situ*.

Quantitative PCR

Quantitative PCR was performed as described [31] using *ltbp3* specific primers LRTF1 (5' – CGCCCAAACAGGCTTGTAGTAGT – 3') and LRTF2 (5' – CACTCTTCGGTGAAAACGG – 3'). *ltbp3* threshold cycles (Cts) were normalized to those for *β-actin* [31] prior to calculating fold differences in expression between experimental groups. For the evaluation of *ltbp3* expression in neural crest depleted embryos, the trunks and tails were omitted from the analysis (by cutting the embryo transversely through the yolk and exclusively analyzing the anterior segments) to ensure that *ltbp3* expression in the notochord did not confound the analysis.

Supplementary (Full) Methods References

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