Semaphorin3f as an intrinsic regulator of chamber-specific heart development

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Abstract

During development a pool of precursors form a heart with atrial and ventricular chambers that exhibit distinct transcriptional and electrophysiological properties. Normal development of these chambers is essential for full term survival of the fetus, and deviations result in congenital heart defects. The large number of genes that may cause congenital heart defects when mutated, and the genetic variability and penetrance of the ensuing phenotypes, reveals a need to understand the molecular mechanisms that allow for the formation of chamber-specific cardiomyocyte differentiation. We find that in the developing zebrafish heart, mRNA for a secreted Semaphorin (Sema), *Sema3fb*, is expressed by all cardiomyocytes, whereas mRNA for its receptor Plexina3 (*Plxna3*) is expressed by ventricular cardiomyocytes. In *sema3fb* CRISPR zebrafish mutants, ventricular chamber development is impaired; the ventricles of mutants are smaller in size than their wild type siblings, apparently because of differences in cell size and not cell numbers, with ventricular cardiomyocytes failing to undergo normal developmental hypertrophy. Analysis of chamber differentiation indicates defects in chamber specific gene expression at the border between the ventricular and atrial chambers, with spillage of ventricular chamber genes into the atrium, and vice versa, and a failure to restrict *bmp4a* mRNA to the atrioventricular canal. The disrupted atrioventricular border region in mutants is accompanied by hypoplastic heart chambers and impaired cardiac function. These data suggest a model whereby cardiomyocytes secrete a Sema cue that, through spatially restricted expression of the receptor, signals in a ventricular chamber-specific manner

to establish a distinct border between atrial and ventricular chambers that is important for functional development of the heart.

Introduction

The heart is the first organ to form and become functional in an embryo, and congenital heart defects affect 9 per 1000 live births¹. Collectively, congenital heart defects involve the irregular formation of heart chambers and/or valves. In vertebrates, chamber morphogenesis follows the twisting, expansion and septation of the linear heart tube into a two- (teleost), three- (amphibian) or four-(mammalian/avian) chambered heart². These cardiac chambers, the atrium and ventricle, differ in their biochemical properties, electrophysiological and contractile capacities, as well as in their transcriptional profiles^{3,4}. What maintains the segregation of the cell populations in the two discrete chambers as the embryonic heart undergoes morphogenesis is unknown.

During development, cells within tissues acquire unique identities, and need to be segregated from one another within subdivisions of the tissue^{5,6}. This is true for example of the developing hindbrain, where the initial expression of genes within adjacent rhombomeres is initially imprecise, and is sharpened as embryogenesis proceeds, producing domains with straight borders⁶. Key in this process are interactions between neighbouring hindbrain cells that involve contact-mediated repellent signaling via Ephrin ligands and Eph receptors. Whether a similar process of refinement of the border occurs between ventricular and atrial chambers of the heart is unknown. Chamber-specific development depends on extrinsic factors, which include Nodal, Notch, Bone morphogenetic protein (Bmp), Fibroblast growth factor (Fgf) and retinoic acid⁷⁻⁹. These signals then pattern the early heart tube to establish secondary localized signaling that spatially restricts the expression of differentiation genes to the specific chambers and valves.

Intrinsic regulators, such as transcription factors, are also necessary for chamber-specific cardiomyocyte development. For example, ventricle specific expression of *Irx4* is necessary in chick for the expression of ventricular Myosin Heavy Chain I (MHC1) and suppression of atrial MHC¹⁰. Further, in mouse PITX2 within the left atrium inhibits the expression of *Shox2* and the specification of the pacemaker cells of the sinoatrial node¹¹. An unanswered question is whether active mechanisms are required to maintain segregation of the two populations of cardiomyocytes once established, especially in light of the disruptive forces of cardiac chamber morphogenesis and heart looping.

One group of molecules known to act as repellents for developing cells of the nervous and cardiovascular systems are the secreted class 3 Semaphorins (Sema3s), which are used as guidance cues for vessels, axons and neural crest cells^{12–15}. Sema3 signaling is mediated through the canonical Plexin (Plxn) receptors and their coreceptors, the Neuropilins (Nrp)¹⁶. Sema3s are implicated in murine cardiovascular development via their regulation of the directed movement of associated cells; SEMA3C¹⁷, SEMA3D¹⁸, SEMA3E¹⁹, and SEMA3G²⁰ pattern embryonic vessels, SEMA3A regulates sympathetic innervation of the heart²¹, and SEMA3C promotes neural crest cell migration into the heart¹⁷. Whether Sema3s play roles in heart formation via the direct regulation of cardiomyocyte development is unknown.

Here, we take advantage of the zebrafish model as a powerful tool to study the development of the heart; the embryos do not require a functional cardiovascular system during embryogenesis²², which allows for the extended characterization of manipulations that are normally fatal. We find that mRNA for the secreted Sema3, Sema3fb, is expressed early in cardiomyocytes during chamber differentiation, while *nrp2b* and *plxna3* receptor mRNAs are expressed by ventricular cardiomyocytes, suggesting spatially restricted Sema3fb signaling

within the developing myocardium. To investigate a role for ventricular cardiomyocyte *Sema3fb* signaling, we generated CRISPR/Cas9 zebrafish *sema3fb* mutants. With either loss of *Sema3fb*, or signaling through its *Plxna3* receptor via morpholino-oligonucleotide knockdown, chamber specific gene expression is either lost from or expands beyond the border between the atrium and ventricle. Accompanying the loss of segregated gene expression between the cardiac chambers is a decrease in the size of both chambers, and significantly impaired cardiac output and function of the larval heart. We propose a model whereby *Sema3fb* signaling ensures segregation of atrial and ventricular cardiomyocyte chambers, a feature that appears critical for chamber development and heart function.

Methods

Zebrafish strains and maintenance

Zebrafish (*Danio rerio*) were maintained according to standard procedure on a 14-h light/10-h dark cycle at 28°C. Embryos were obtained by natural spawning, raised in E3 medium supplemented with 0.25 mg/L methylene blue, and staged by convention^{23,24}. To permit exact developmental staging, with all embryos synced, adult zebrafish spawned for 10 minutes only before embryo collection. Dishes were screened at 6 hours post fertilization (hpf) to remove embryos that showed delayed embryogenesis or unfertilized eggs. We used *sema3fb*^{ca305} and *sema3fb*^{ca306} genetic mutants. These lines were maintained as heterozygotes for all initial experiments, and thereafter maintained as homozygotes and wild type (WT) siblings for future embryo collection. The *Tg(-6.5kdr1:mCherry)*^{ci5} line was used as an outcross for *sema3fb*^{ca305} mutants to label endothelial cells²⁵. Sex was not a variable in these studies as zebrafish cannot be

sexed as embryos. The University of Calgary Animal Care Committee approved all procedures (#AC19-0114).

Establishment of *sema3fb* mutant lines

To generate genetic knock-outs by using the CRISPR/Cas9 system a sgRNA (5' - GAAGGACAAGAAGACCCGCG) targeting *sema3fb* exon 2 was selected following CHOPCHOP query²⁶ and analysis of secondary structure using Vienna RNAfold Prediction (rna.tbi.univie.ac.at). sgRNA template generation and transcription was carried out in accordance to the protocol described previously²⁷. 1-cell stage embryos were injected with a 1 nl mix of approximately 56-60 pg sgRNA and 190 pg *cas9* mRNA (Addgene plasmid #47322). Mosaic embryos were raised to adulthood and crossed with Tupel Long fin (TL) fish to identify founders. To genotype, caudal fin clippings were collected from tricaine methanesulfonate (MS-222; 160mg/L) anesthetized adults. Genomic DNA extraction was performed²⁸ and amplified by PCR using primers (Forward: 5'- ATTGCCCCACAAAATAACATTC; Reverse: 5' - GTCTACTCTGTGAATTTCCCGC) around the expected mutation site. Amplicons were sequenced at University of Calgary DNA Core facility for definitive genotype confirmation.

RNA extraction and quantitative real time PCR

Total RNA from embryos was prepared using the TRIzol reagent (Invitrogen) extraction method²⁹. Briefly, 5 embryos or embryonic hearts at specific developmental ages were collected in Trizol and homogenized using a 26½ gauge syringe. First strand cDNA was made using the Superscript II RT-PCR (Invitrogen, 11904-018) protocol with 100 ng of total RNA for conversion. Each RT-qPCR reaction had a 10 µl final volume containing 0.5 µl of cDNA, 500

1 nM of each primer and 5 µl of SYBR Green QuantiFast RT-qPCR master mix (Qiagen). Primers
2 used were: *sema3fb* (F: 5'- AGTGACGCATATGGCTCTGC; R: - 5'-
3 AGGAAGCCTCTTCTGCGAGG); *bmp4a* (F: 5' – GACCCGTTTTACCGTCTTCA; R: 5'-
4 TTTGTCGAGAGGTGATGCAG) (Morris et al., 2011); *tbx5a* (F: 5'-
5 AACCATCTGGATCCCTTCG; R: 5'- TGTTTTTCATCCGCCTTGAC). An Applied Biosystems
6 QuantiStudio 6 Real-Time PCR system was used, and gene expression was normalized relative
7 to reference gene *β-actin* (F: 5'-GCAGAAGGAGATCACATCCCTGGC; R: 5'-
8 CATTGCCGTCACCTTCACCGTTC). Reactions were performed in three technical replicates,
9 and all results made from 3-4 independent biological replicates.

***in situ* hybridization**

11 Digoxigenin RNA probes were synthesized as described previously³⁰. Probes were generated
12 from plasmid templates or via PCR products containing an RNA polymerase binding sequence
13 on the reverse primer. Probes used included: *bmp4a* (5'- CTGCCAGGACCACGTAACAT; 5'-
14 GAAATTAATACGACTCACTATAGGGTGGCGCCTTTAACACCTCAT); *crip2* (5'-
15 GCCCCAGATGCAGCAAGAAG; 5'-
16 TCATTTAGGTGACACTATAGACCAGCACCAGTCACAAACACC); *irx1a* (5'-
17 GAGAACAAGGTGACCTGGGG; 5'-
18 GAAATTAATACGACTCACTATAGGGTGAAGAGGACGAAACGACGA); *ltbp3a* (5'-
19 ACTCACCTTTAGTGCAGCCC; 5'-
20 GAAATTAATACGACTCACTATAGGGCTTCAAATGGGCGCAAACCC); *myh6* (5'-
21 GGAGTACGTGAAGGGGCAAA; 5'-
22 GAAATTAATACGACTCACTATAGGGGCTCGTCCCGAAATGAATGC); *myh7* (5'-
23 GCAACTTGGTGAGGGAGGAA; 5'-

GAAATTAATACGACTCACTATAGGGAGCAAGCTTACGGCCTCTTT); *nrp2b* (5'-TCCGGGATGGGAACTCAGAT; 5'-GCATTTAGGTGACACTATAGAATCGGCCGTCATAGAAGCTG); *plxna3* (pCRII, linearized with EcoRV); *sema3fb* (pCR4, linearized with NotI); *tbx5a* (pCRII, linearized with EcoRV). Whole mount *in situ* hybridization was performed as described previously with some minor modifications³⁰. We omitted steps with gradient changes in hybridization buffer, and the 2xSSC step was carried out at 70°C and 0.2 x SSC at 37°C. Embryos were incubated directly in anti-DIG-AP, Fab fragment antibody (Roche) solution in blocking buffer at room temperature for 2 hours with no blocking step. NBT/BCIP (Roche) was used at a 2.5/3.5µl/ml ratio to stain embryos. Stained embryos were fixed in 4% paraformaldehyde (PFA), and washed in PBS with 0.1% Triton X-100 (PBST). When directly comparing *in situ* hybridization signals between genotypes, all embryos were processed in the same tube.

Immunolabeling and Imaging

Embryos were fixed overnight in 4% PFA in PBS and processed for whole mount immunostaining. Briefly, samples were blocked in PBST with 10% bovine serum albumin and 2% normal sheep serum for 30 minutes and transferred to primary antibody solution in 1/10 blocking buffer overnight at 4°C. Samples were then washed with PBST and incubated in secondary antibody (Alexa Fluor 488/555 rabbit/mouse) supplemented with 5 mg/ml Hoechst in PBST for 45 minutes. Embryos were fixed and imaged by confocal microscopy. The following primary antibodies were used: MF20 (1:500, Developmental Studies Hybridoma Bank), pHH3 (1:500, Millipore), zn8 (DM-GRASP; 1:500, Developmental Studies Hybridoma Bank). ApopTag Peroxidase In Situ Apoptosis Detection kit (Millipore) was used to detect apoptotic cells in whole mount embryos, with overnight diaminobenzidine staining. For confocal

microscopy, embryos were imaged with a 10 or 20x objective on a Zeiss LSM 700 microscope after mounting in 0.8-1% low melting point agarose (Invitrogen) on a glass bottom dish. Optical slices were taken at intervals from 1-5 μ m and subjected to 2 times averaging. Z-stacks were processed in Zen Blue as maximal projections and compiled using Adobe Photoshop 2020.

Heart Function and Morphology Analysis

Embryos (72 hpf) were anaesthetized with minimal MS-222 and positioned on their right side laterally on a glass slide in a water droplet. Video images were taken 30 frames/second for 15 secs using a Leica DM5500 B microscope equipped with a Leica DFC365 FX. Heart rate was determined in a 15 sec window. Measurements³¹ were made from frame stills during systole and diastole across three heart beats using ImageJ. Briefly, the following equations were used²:

Ventricle volume = $(0.523) (\text{Ventricle Width}) (\text{Ventricle Length})$; Fractional Shortening = $(100) (\text{Ventricle Width Diastole} - \text{Ventricle Width Systole}) / \text{Ventricle Width Diastole}$; Stroke Volume = Ventricle Volume Diastole – Ventricle Volume Systole. Ejection Fraction = $(100) (\text{Stroke Volume} / \text{Ventricle Volume Diastole})$. Cardiac Output = (Heart Rate) (Stroke Volume).

Hematoxylin and Eosin (H&E) staining

H&E staining of JB-4 plastic sections was carried out as described previously³² with the following modifications; Acid and base washes were omitted, Harris Modified Hematoxylin Solution (Sigma) and Eosin Y (EMD) were used, and regular tap water replaced prescribed substitute. Slides were air dried for 15 mins³³ and coverslipped using Permount (Fischer Scientific). Myocardial ventricle wall thickness for each embryo was the average of measurements made at two points along the anterior-posterior axis from 1-2 sections stained by H&E.

Cardiomyocyte measurements

Morphometries (area and perimeter) of confocal imaged, Zn8-immunolabelled cardiomyocytes were measured in ImageJ at 48 hpf. Cells measured had clearly visible outlines. Of note, only cells central to the outer curvature of the ventral ventricle were assessed. Circularity was used to distinguish cellular morphologies in which 1 represents a perfect circle. This was calculated by the following formula: $\text{Circularity} = 4 \pi \text{Area} / \text{Perimeter}^2$. Measurements were averaged from 8-12 cells per embryo. Additionally, projected confocal MF-20-immunolabeled images were analyzed using ‘RGB Measure’ tool on ImageJ across the entirety of the heart, values averaged for all samples, and represented as an intensity plot across distance.

Sema3f Overexpression and Knockdown

For heart specific overexpression, *sema3fa* was amplified from Image Clone 9038816 using primers that incorporate *attb1/b2* recombination sites (5’-GGGGACAAGTTTGTACAAAAAGCAGGCTTCACCATGCAGGGAGCCGGGACTTTGGTG-3’ and 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTTTGTCTCAGCCATGCTGCTCTGCTC-3’) and inserted into pDONR221 to create a *pME-sema3fa* vector. Three way Tol2 gateway cloning³⁴ was used to insert *sema3fa* downstream of the cardiomyocyte *myl7* promoter³⁵ giving *myl7:sema3fa:p3E-MTpA* in Tol2 with *pDest:mKate*³⁶ as the transgenesis marker. One cell-stage zebrafish embryos were injected with a solution consisting of 12.5-25 ng/μl plasmid with or without 25 ng/μl *transposase* mRNA. At 48 hpf the embryos were checked for mKate eye expression and fixed for *irx1a* and *bmp4a* *in situ* hybridization as well as anti-myc immunohistochemistry. For knockdown of *sema3fb* we injected either 1 or 3 ng/ml of an antisense morpholino oligonucleotide that targets the ATG start site of *sema3fb* (CATAGACTGTCCAAGAGCATGGTGC, Gene

Tools LLC, Corvallis, OR, USA) into 1-cell zebrafish embryos. For *plxna3* and *nrp2b* knockdown we used previously characterized MOs³⁷. At 48 hpf, embryos were scored for edema and fixed for *in situ* hybridization analyses.

Statistics

All data sets for qualitative scoring or quantitation were analyzed in a blinded fashion. Sample size calculations were not performed because we characterized mutants, and thus had no prediction as to the likely size of effects to use in power calculations. Results are expressed as mean $\pm$ standard error the mean (SEM) unless otherwise indicated. All statistical analysis was performed using Prism 8 software (Graph Pad). An unpaired, non-parametric Mann Whitney U test was used for comparisons of two samples, and a One-Way ANOVA with a Kruskal-Wallis test allowed for comparisons between multiple samples.

Results

***sema3fb* is expressed by the cardiomyocyte progenitor cells of the developing heart**

To elucidate the spatial and temporal expression of *sema3fb* in the zebrafish heart we performed whole mount RNA *in situ* hybridization (ISH) on embryos from 10-72 hours post fertilization (hpf). In zebrafish, cardiac progenitors of the atrial and ventricular chambers are bilaterally specified by 5 hpf³⁸. The progenitors then condense at the midline and undergo a series of movements to produce a linear tube of differentiating myocardium that surrounds the underlying endocardium^{38,39}. This tube starts pumping between 24-26 hpf⁴⁰. By 48 hpf, the two-chambered heart is discernable by the constriction of the atrioventricular canal^{38,41}. *sema3fb*

transcript was first detected at 10-18 hpf in a bilateral fashion in the presumptive cardiac progenitor fields, as revealed by the cardiomyocyte progenitor marker *hand2* (Suppl Fig. 1B). By 24 hpf, *sema3fb* was expressed by cardiomyocyte progenitors through the entire linear heart tube (Figure 1A), as evidenced by ISH signal similar to that seen with the pan-cardiomyocyte marker *cardiac troponin T type 2a (tnnt2a)* (Figure 1B)⁴². A thin transverse plastic section of *sema3fb* whole mount ISH on a *Tg(flk:EGFP)* background, where GFP labels the endocardial cells that line the ventricles, revealed that *sema3fb* was expressed not by GFP positive cells but the cardiomyocytes of both atrial and ventricular chambers (Figure 1C-E). *sema3fb* remained expressed in both the ventricle and the atrium over the period that the heart undergoes rightward looping and chamber morphogenesis (24-48 hpf) (Figure 1F). Weak expression persisted throughout the ventricular and atrial myocardium of the 72 hpf heart (data not shown). These data indicate that mRNA for a secreted Sema is expressed by cardiomyocytes at the time they undergo differentiation, and agree with RNAseq analysis of FACs-isolated zebrafish cardiomyocytes, where *sema3fb* mRNA is enriched in the heart over the rest of the embryo⁴³. The presence of *sema3fb* in cardiomyocytes suggests a role for Sema3fb in heart development in a novel heart-intrinsic manner.

To assess the importance of Sema3fb for heart development and function we used CRISPR/Cas9 gene editing technology²⁷ to generate *sema3fb* genetic mutants. A specific guide RNA (sgRNA) targeting exon 2 (Figure 1H) was co-injected with *cas9* mRNA at the one cell stage and a single founder was recovered that housed two allelic variants; a 19 base pair (bp) deletion and a 10 bp insertion. Both lines were propagated from this founder to generate stable homozygous viable generations, alongside wild type (WT) siblings. RT-PCR analysis of mRNA isolated from 24 hpf embryos indicated that the 19 bp deletion mutant (hereafter *sema3fb*^{ca305})

lacked exon 2 that contains the ATG start site, presumably because the deletion at the end of exon 2 disrupted a key splice site (Figure 1G). Indeed, sequencing of the PCR product generated with primers in exon 1 and exon 3 indicated that exon 1 was spliced in frame with exon 3 to give an mRNA that if translated would produce a protein missing the first 62 amino acids (aa), including the signal sequence (amino acids 1-23) required for protein secretion and the first 14 aa of the SEMA domain that is necessary to elicit intracellular signaling⁴⁴ (Figure 1H). Software could not identify another signal sequence in the predicted translated protein, thus in the *sema3fb*^{ca305} allele the C-terminal truncated Sema3fb would not be secreted from cardiomyocytes. RT-PCR and sequencing analysis indicated that exon 2 was included in the *sema3fb* mRNA product in the 10 bp insertion allele (hereafter *sema3fb*^{ca306}) (Figure 1G). The *sema3fb*^{ca306} allele is predicted to produce a protein that is 58 aa in length and is prematurely truncated within the SEMA domain (Figure 1H). Of note, a commercial antibody against mouse SEMA3F failed to distinguish between multiple Sema3s in zebrafish (data not shown). Importantly, while injection of an antisense morpholino oligonucleotide (MO) against the ATG start site of *sema3fb* produced a similar heart edema when injected at the one cell stage in WT embryos as seen in the *sema3fb*^{ca305} allele, there was no greater incidence of severe edema seen when the MO was injected into the *sema3fb*^{ca305} allele (Suppl Figure 1A). The MO data argue strongly for the specificity of the heart phenotype in the *sema3fb* mutants being due to the loss of Sema3fb. Of note, *sema3fb* mRNA was not detected in the 48 hpf WT and *sema3fb* mutant heart (Suppl Figure 1C), arguing that there is no compensation for the loss of Sema3fb by upregulation of Sema3fa expression.

Embryos that lack Sema3fb exhibit cardiac edema

To examine potential heart defects with the loss of *Sema3fb*, heterozygous adults were incrossed, and progeny raised to homozygosity and incrossed with their respective genotypes (*sema3fb*^{+/+}, *sema3fb*^{ca305}, *sema3fb*^{ca306}). At 72 hpf, embryos were indistinguishable by gross size, but homozygous embryos exhibited edema (Figure 2A) that presented in both allelic variants at 48 hpf (8.3% *sema3fb*^{+/+}, 91.7% *sema3fb*^{ca305}, 76.7% *sema3fb*^{ca306}, N=3, n=60 each genotype) and 72 hpf (5.0% *sema3fb*^{+/+}, 91.7% *sema3fb*^{ca305}, 41.7% *sema3fb*^{ca306}, N=3, n=60 each genotype) (Figure 2B). Most mutant embryos exhibited edema, though the severity of the defect varied. Generally, embryos with edema developed to adulthood, though a small proportion (~10%) of homozygous embryos did not survive past 5-7 days post fertilization (dpf), and exhibited severe cardiac edema (Figure 2C,D), a hypoplastic ventricle and a linear arrangement of the heart tube (Figure 2C'). All analyses were carried out on embryos in which the pericardial edema was of average extent, with lethal phenotypes excluded so as not to confound data interpretation. Of note, heterozygous *sema3fb*^{ca305} and *sema3fb*^{ca306} embryos also exhibited a spectrum of edema, ranging from no edema to severe edema, suggesting that genetic dosage influences the phenotype (data not shown). Additionally, as the phenotypes between the two allelic variants were highly comparable, we focused on the *sema3fb*^{ca305} mutant in our analyses, and confirmed key findings in the *sema3fb*^{ca306} allele. From hematoxylin and eosin (H&E) sections we found that the thickness of the ventricle myocardium at 72 hpf was reduced significantly ($6.3 \pm 0.5 \mu\text{m}$, standard error of the mean (s.e.m.) here and throughout the paper, unless otherwise indicated; Mann Whitney U, p=0.024, N=2, n=6) in *sema3fb*^{ca305} as compared to WT ($9.4 \pm 0.7 \mu\text{m}$; N=2, n=3) hearts (Figure 2E), suggesting that in addition to edema, myocardial development was impaired.

***sema3fb* mutants have impaired cardiac function**

By 72 hpf in zebrafish, cardiac function is considered rhythmic and the circulatory system relatively mature⁴⁵. To determine whether the edema in *sema3fb*^{ca305} mutants resulted from impaired cardiac function, 72 hpf hearts were imaged by live video microscopy, and measurements of the ventricle were made at end-diastole and end-systole (Figure 3A-B). Ventricle volume was reduced significantly during diastole (0.50 ± 0.03 nL; $p=0.0027$) and systole (0.32 ± 0.02 nL; Mann Whitney U, $p=0.0063$) in *sema3fb*^{ca305} mutants (N=3, n=20) as compared to WT siblings (diastole, 0.68 ± 0.05 nL; systole, 0.39 ± 0.02 nL; N=3, n=12) (Figure 3C-D). Additionally, mutants had a small but significantly slowed heart rate (WT 151.7 ± 1.4 beats/min, N=3, n=12; *sema3fb*^{ca305} 143.0 ± 1.5 beats/min, N=3, n=20; Mann Whitney U, $p=0.0008$) (Figure 3E). Cardiac output (WT 44.5 ± 7.4 nL/min, N=3, n=12; *sema3fb*^{ca305} 25.7 ± 2.6 nL/min; Mann Whitney U, $p=0.021$) and stroke volume (WT 0.29 ± 0.05 nL, N=3, n=12; *sema3fb*^{ca305} 0.18 ± 0.02 nL, N=3, n=21; Mann Whitney U, $p=0.04$) (Figure 3F-G) were also reduced significantly in mutants as compared to WT, while ejection fraction (WT $40.5 \pm 3.7\%$, N=3, n=12; *sema3fb*^{ca305} $35.4 \pm 3.0\%$, N=3, n=20; Mann Whitney U, $p=0.45$) and fractional shortening (WT $17.6 \pm 2.0\%$, N=3, n=12; *sema3fb*^{ca305} $13.8 \pm 1.6\%$, N=3, n=20; Mann Whitney U, $p=0.17$) (Figure 3H,I) were unchanged. Overall, the heart of the *sema3fb* mutant embryo was impaired significantly in function.

Early heart development occurs normally in the *sema3fb* mutants

The impaired function of the 72 hpf *sema3fb* mutant heart suggested a defect in heart development, which could include problems with cardiac progenitor cell specification and differentiation, morphogenesis of the heart tube, chambers and atrioventricular canal, formation

of the endocardial heart valves, or the establishment of normal cardiac function⁴⁶. To begin to address the underlying biology, we examined whether there were defects in early cardiac development, since *sema3fb* is expressed early in the bi-lateral heart fields (Suppl Figure 1B). Expression of *fgf8a*, a marker of the heart fields⁴⁷, was not affected in the *sema3fb* mutants (Suppl Figure 2A-C), nor was its expression impacted elsewhere in the embryo, including in the brain, somites and tailbud region (Suppl Figure 2D-F), suggesting that early patterning was not disrupted by the *sema3fb* mutation. Next, we assessed the heart at 24 hpf by whole mount ISH for the cardiomyocyte marker *tnnt2a*, and for *myosin heavy chain 7 (myh7)*⁴⁸ and *myosin heavy chain 6 (myh6)*⁴⁹ to label ventricular and atrial cardiomyocytes, respectively (Suppl Figure 3). *sema3fb*^{ca305} and *sema3fb*^{ca306} hearts were generally in a cone/condensed state at 24 hpf (Suppl Figure 3B,C,F,G) and had not yet elongated into the linear tube of WT hearts^{50,51} (Suppl Figure 3A,E,D,H,L). Nonetheless, two hours later tube formation had occurred in *sema3fb* mutants; 80% of *sema3fb*^{ca305} (N=1, n=8/10) and 90% of *sema3fb*^{ca306} (N=1, n=9/10) mutants exhibited elongated heart tubes at 26 hpf (Suppl Figure 3N,O), comparable to the numbers observed in WT siblings (N=1, n=9/10) at 23 hpf (Suppl Figure 3M,R). Notably, the cranial ganglia that emerge immediately adjacent to the heart developed normally in aged-matched embryos (Suppl Figure 3P,Q). Overall these data argue that heart morphogenesis is delayed only slightly (by 2-3 hours) by the loss of *Sema3fb*, with cardiomyocyte marker expression present at 24 hpf.

Heart valve development disrupted slightly with loss of *Sema3fb*

Edema can result from a failure of valves to form between the chambers that results in retrograde flow of blood and fluid build-up⁵². Valves were present, however, when assessed in H&E-labeled 72 hpf *sema3fb*^{ca305} heart sections (Figure 4A-B). To confirm these data, we bred

the *sema3fb*^{ca305} allele onto the *Tg(kdrl:mCherry)* line, in which the endocardial cells that make up the valves⁵² are mCherry positive at 72 hpf²⁵. We visualized the heart by immunolabeling with the cardiac muscle marker MF20⁵³. In both the WT and *sema3fb*^{ca305} hearts, valves were present at the border between the atrium and ventricle (Figure 4C,D), and were of similar lengths (N=2; WT 61.6 $\mu\text{m} \pm 2.5 \mu\text{m}$, n=10 embryos; *sema3fb*^{ca305} 60.1 $\mu\text{m} \pm 3.8 \mu\text{m}$, n=12 embryos; Mann Whitney U, p=0.72) (Figure 4J). The intensity of the mCherry label in the *sema3fb*^{ca305} valves, however, was reduced as compared to WT (Figure 4C',D'). Further, the distribution of the valves differed between the genotypes, with the valves in WT hearts roughly symmetrically distributed on either side of the constriction between the atrial and ventricular chambers, but preferentially located on the ventricular side of this border in mutants (Figure 4K).

To assess whether early development of the endocardium, from which valves form, occurred normally in the absence of *Sema3fb*, we examined the expression of the early endocardial marker *nfatc1*^{54,55}. While *nfatc1* ISH signal was present at comparable levels in WT and mutant hearts at 28 hpf, the domain in *sema3fb*^{ca305} embryos (84.3 $\mu\text{m} \pm 9 \mu\text{m}$, N=2, n=15 embryos) was significantly shorter than in WT (205.7 $\mu\text{m} \pm 7 \mu\text{m}$, N=2, n=13; Mann Whitney U test, p<0.001) (Figure 4E,F,L). Yet, *nfatc1* mRNA was localized preferentially to the atrioventricular constriction at 48 hpf in both genotypes (N=2, WT, n=21/22 embryos; *sema3fb*^{ca305}, n=16/18 embryos) (arrowhead, Figure 4G-H), and expression of the *vegfr2* endothelial cell marker was similar in the two genotypes (Figure 4I). These data suggest a delay in endocardial development, similar to what is seen with heart tube elongation (Suppl Fig. 3), but no gross defect in the initial formation of this tissue in *sema3fb*^{ca305} embryos.

Heart chamber size is decreased in *sema3fb* mutants

Heart chamber morphogenesis is complete by 48 hpf⁵⁰, and thus a discernable defect in the morphology of the heart at this time-point would be expected to manifest as a functional deficit by 72 hpf. To determine the state of chamber development at 48 hpf we used the *tnnt2a* marker, which labels the entire heart. Looped hearts with a morphologically evident ventricle and atrium were present across all genotypes (Figure 5A-C). Measurements of the antero-posterior length of the *tnnt2a*-labelled heart (Figure 5D), however, revealed that the hearts of both *sema3fb*^{ca305} ($122.8 \pm 5.0 \mu\text{m}$, N=3, n=16; Kruskal-Wallis, Dunn's multiple comparisons, $p < 0.0001$) and *sema3fb*^{ca306} ($135.4 \pm 4.5 \mu\text{m}$, N=3, n=16; Kruskal-Wallis, $p = 0.0016$) mutant embryos were significantly shorter than WT ($163.8 \pm 4.0 \mu\text{m}$, N=3, n=18).

To determine if one or both cardiac chambers were affected, we used whole mount ISH at 48 hpf for *myh*⁷⁴⁸ and *myh*⁶⁴⁹ to label ventricular and atrial cardiomyocytes, respectively. Both cardiac chambers were smaller in mutants as compared to WT. The long axis of the *sema3fb*^{ca305} ($121.5 \pm 3.3 \mu\text{m}$, N=3, n=27; Ordinary one-way ANOVA, Tukey's multiple comparison test, $p = 0.009$) and *sema3fb*^{ca306} ($122 \pm 3.1 \mu\text{m}$, N=3, n=26; Ordinary one-way ANOVA, $p = 0.013$) ventricles was significantly shorter than in WT ($138.4 \pm 5.1 \mu\text{m}$, N=3, n=28) (Figure 5E-H), as confirmed by measurements of the ventricle length during diastole (WT $203.9 \pm 6.7 \mu\text{m}$, n=12; *sema3fb*^{ca305} $190.5 \pm 2.3 \mu\text{m}$, n=20; Mann Whitney U, $p = 0.048$) and systole (WT $175.6 \pm 4.2 \mu\text{m}$, n=12; *sema3fb*^{ca305} $163.3 \pm 3.4 \mu\text{m}$, n=20; Mann Whitney U, $p = 0.031$) in live embryos viewed in brightfield (Suppl Figure 4). Additionally, the *myh*⁶⁺ atria were significantly smaller in area in the *sema3fb*^{ca305} ($8,290 \pm 360 \mu\text{m}^2$, N=3, n=19; Kruskal-Wallis, $p = 0.0001$) and *sema3fb*^{ca306} ($6,760 \pm 450 \mu\text{m}^2$; N=2, n=7 Kruskal-Wallis, Dunn's multiple comparisons, $p < 0.0001$) hearts than in WT ($11,390 \pm 460 \mu\text{m}^2$; N=3, n=19) (Figure 5I-L). Ultimately, these

data suggest that with the loss of *Sema3fb* both ventricle and atrium are specified, but the chambers are smaller in size than in their WT counterparts.

The border between the ventricle and atrium appears disrupted with loss of *Sema3fb*

Interestingly, close inspection revealed that borders of the domains of expression of atrial and ventricular specific *myosins* at the atrioventricular junction were disrupted in *sema3fb* mutants. In the vast majority of WT hearts (N=4, 31/33 hearts), the *myh7*-expression border aligned with the constriction between the ventricle and atrial chambers (Figure 5F). In contrast, in both the *sema3fb^{ca305}* (N=4, n=27/28 hearts; Figure 5F) and *sema3fb^{ca306}* (N=2, n=11/12; Figure 5G) heart, expression of *myh7* was low or absent from the atrioventricular band (yellow arrowheads, Figure 5F,G), such that the posterior border of the high *myh7* expression domain sat anterior to the band. We quantitated this phenotype by measuring the *myh7* negative area between the atrioventricular constriction and the posterior domain of solid *myh7* expression (Figure 5Q), and found a significantly larger non/low-expressing domain in both mutants than in WT (WT $383 \pm 46 \mu\text{m}^3$, n=28 embryos; *sema3fb^{ca305}* $1205 \pm 86 \mu\text{m}^3$ n=26; *sema3fb^{ca306}* $1516 \pm 177 \mu\text{m}^3$, n=18; p<0.0001 Kruskal-Wallis test, Dunn's multiple comparisons). Additionally, while the border of the *myh6* atrial marker expression domain was generally smooth in WT hearts (N=3, n=22/26 hearts; Figure 5I), it was often disorganized in both the *sema3fb^{ca305}* (N=3, n=23/31) and *sema3fb^{ca306}* (N=1, n=4/7) hearts (Figure 5J,K). Furthermore, in most WT hearts the anterior border of *myh6* expression aligned with the morphological constriction between the atrium and ventricle (n=20/26), but often extended a small extent past this border and into the ventricle in *sema3fb^{ca305}* (N=4, n=23/31) and *sema3fb^{ca306}* (N=1, n=6/7) hearts (Figure 5I-K). Of note, in younger 36 hpf hearts this antero-wards extension of the *myh6* domain past the constriction was

present in both WT and *sema3fb*^{ca305} genotypes (Figure 5M,N), suggesting that there was a failure to resolve to the mature border in mutants. The defect in chamber size was still present in *sema3fb* mutants at 72 hpf, as evident by *myh6* expression in the atria (Figure 5O,P), and confirmed by quantitation of the area of the atrial *myh6*⁺ expression domain in WT vs. mutants (N=2; WT, 10,518 ± 618 μm³, n=18 embryos; *sema3fb*^{ca305} 7929 ± 472 μm³, n=27; p=0.0021 Mann Whitney U test) (Figure 5R). These data indicate that with *Sema3fb* loss there is disruption of the expression of mRNA for atrial and ventricular myosins at the border between the two chambers. We also asked if expression of a non-myosin ventricular chamber specific gene was disrupted at the atrioventricular junction. Normally, *tnnt2a* mRNA is expressed at higher levels in the ventricle than atrium, as evidenced in WT hearts by a sharp drop in mRNA levels in the atrium relative to the ventricle (Figure 6A). This drop was less dramatic in both *sema3fb* mutant alleles, and an obvious border between expression in the two chambers was lacking.

Possibly, the disrupted chamber-specific myosin expression in mutants was a feature of altered chamber-specific cardiomyocyte differentiation. Thus, we looked at the expression of the ventricle-specific homeobox-containing transcription factor, *iroquois1a* (*irx1a*)⁵⁶ at 48 hpf. Interestingly, despite the reduction of ventricular marker *myh7* mRNA at the atrioventricular junction, *irx1a* mRNA expression in mutant and WT hearts was similar, with expression extending through the atrioventricular canal in both genotypes (Figure 6B,C; N=2; WT, n=15/15 hearts; *sema3fb*^{ca305} n=16/16). In agreement, the high-to-low ventricle-to-atrium gradient of *T-box* transcription factor *tbx5a*⁵⁷ mRNA was unchanged between WT (N=1, n=9) and *sema3fb*^{ca305} (N=1, n=7) embryos (Figure 6D,E), as verified by RT-qPCR (-/- 0.93 ± 0.3% (standard deviation) fold change vs. WT, N=3; Mann Whitney U, p=0.7; Figure 6I). These data indicate that the ventricular differentiation program is not affected globally by *Sema3fb* loss.

Because of the altered expression of *myh7* at the AVC, we next asked whether *bone morphogenetic protein 4a* (*bmp4a*) expression at the AVC was regulated appropriately. *bmp4a* is expressed initially throughout the antero-posterior length of the myocardium and then becomes largely restricted to the AVC by 37 hpf⁵⁸. By 48 hpf, we found the strongest *bmp4a* mRNA signal was in the AVC region in almost all WT (N=4; 96.7%, n=29/30), and *sema3fb*^{ca305} (N=4; 91%, n=41/45) hearts (Figure 6F,G). In the majority of WT hearts, *bmp4a* was restricted to the AVC alone (N=4; 70%, n=21/30), with a smaller number of hearts showing additional ISH signal throughout the ventricle (20% n=6/30) or in the atrium (13.3%, n=4/30). In contrast, in mutant hearts *bmp4a* mRNA often continued to be expressed throughout the ventricle (71.1%, n=32/45) and/or in the atrium close to the AVC (53.3% n=24/45). RT-qPCR verified that at 48 hpf *bmp4a* levels were upregulated in the *sema3fb*^{ca305} hearts (-/- 1.79 ± 0.33% (standard deviation) fold change vs. WT, N=5; Mann Whitney U, p=0.036) (Figure 6J). Furthermore, quantitation revealed a significantly larger *bmp4a* expression domain in *sema3fb* mutant hearts than WT (Figure 6K; N=4; WT 1770±145 μm³, n=30; *sema3fb*^{ca305} 4109±332 μm³ n=45; p<0.0001, Mann-Whitney U test). By 72 hpf, mutant hearts still exhibited a handful of *bmp4a*-expressing ectopic cells within the ventricle (60% n=9/15) or atrium (73% n=11/15), though *bmp4a* expression was concentrated mainly at the AVC, as was the case in all WT hearts (n=24/24) (Figure 6H,I).

Sema3f may be required either to inhibit expression of *bmp4a* throughout the ventricle or to mark the border between atrium and ventricle and identify the domain to which *bmp4a* expression should resolve. We used Sema3f overexpression to address these two possibilities. If the former was true, we expected to see a knockdown of *bmp4a* levels in hearts overexpressing Sema3f. In the latter scenario, misexpression of Sema3f would disrupt the border and a

broadened *bmp4a* AVC expression domain might result. To overexpress Sema3f in developing cardiomyocytes we injected a Tol2 *myl7:sema3f-myc* construct into embryos at the one-cell stage along with *transposase* mRNA, with injection of the plasmid alone serving as control. We tried but failed to clone *sema3fb*, however, a full-length cDNA clone of zebrafish *sema3fa* was available. Sema3fa and Sema3fb paralogs share 83% amino acid identity, and *sema3fa* or *sema3fb* mRNA both result in delayed neutrophil recruitment in a zebrafish model of inflammation⁵⁹. Thus, we expected Sema3fa to behave like Sema3fb. At 48 hpf, the *sema3f*-expressing fish were processed by whole mount ISH for *bmp4a* mRNA. Notably, Sema3f was expressed in a mosaic fashion in both the atrium and ventricle, as visualized by immunolabeling for the myc-tagged Sema3f (Figure 6L). Sema3f overexpression produced a similar *bmp4a* phenotype to that seen with loss of Sema3fb. The control and Sema3f-expressing hearts both exhibited enhanced expression of *bmp4a* at the AVC by 48 hpf (Control, 100% n=20/20; Sema3f overexpression, 97% n=35/36), however, *bmp4a* ISH label expanded beyond the AVC with Sema3f overexpression (arrowheads, Figure 6M). *bmp4a* mRNA was present only within the AVC in most control hearts (75%, n=15/20), but this was true in a small number of *sema3f*-overexpressing hearts (25%, n=9/36), where the *bmp4a* domain spread beyond the AVC into the atrium and/or ventricle. In support, the area of the *bmp4a* mRNA domain was significantly smaller in the Sema3f-overexpressing hearts than control (Figure 6O). These data argue that Sema3fb does not directly regulate *bmp4a* expression, but that *bmp4a* expression is altered as a downstream consequence of the loss of Sema3fb signaling. The fact that both loss and gain of Sema3f function produced a similar phenotype argues that normally Sema3fb signaling needs to be strictly regulated, and that either too little or too much signaling disrupts normal development of the border between the ventricular and atrial chambers.

To confirm at the protein level that there was disruption of the border between the two heart chambers in *sema3fb* mutants, we used two antibodies that label proteins that are expressed at higher levels by ventricular than atrial myocardium at 48 hpf: MF20 labels cardiac muscle and is commonly used as a ventricular marker in zebrafish^{57,58}, and an antibody against the cell-surface adhesion molecule DM-GRASP that labels the surface of cardiomyocytes⁶⁰. Whole mount immunostaining, followed by confocal microscopy, revealed clearly separate atria and ventricles in WT hearts at 48 hpf (N=1, n=5/5 MF20; N=3, n=11/12 DM-GRASP, Figure 7A,C). In contrast, the distinct border between the chambers was disrupted in at least 83% of mutants (N=1, n=8/9 MF20; N=3, n=10/12 DM-GRASP, Figure 7B,D). This loss of a sharp ventricle to atria border was evident with the quantitation of the average intensity of MF20 immunolabel over the antero-posterior axis of the heart (WT n=5, *sema3fb*^{ca305} n=9; Figure 7E).

A decrease in cardiomyocyte size explains the small ventricles observed in *sema3fb* mutants

The small size of the ventricles and atria of *sema3fb* mutants at 48 hpf could be due either to fewer cardiomyocytes or a reduction in cardiomyocyte size. While our data indicate that cardiomyocyte progenitor cells are specified and differentiate in the absence of Sema3fb, subsequently less proliferation and/or increased apoptosis could reduce their numbers. Similar numbers of mitotically active cardiomyocytes, however, were labelled with an antibody against phosphorylated histone H3 (pHH3) in 48 hpf WT and *sema3fb*^{ca305} hearts (WT 1.31 ± 0.2 cells, N=3, n=16; *sema3fb*^{ca305} 1.44 ± 0.2 cells, N=3, n=27; Mann Whitney U, p=0.71) (Figure 7A,B,F). Furthermore, no significant changes were observed at 48 hpf in the numbers of TUNEL positive apoptotic cardiomyocytes in WT and *sema3fb*^{ca305} embryos (WT 0.62 ± 0.3 cells, N=2, n=8; *sema3fb*^{ca305} 0.62 ± 0.3 cells, N=2, n=8; Mann Whitney U, p>0.99) (Figure 7G). In support

of these data, similar numbers of MF20+ cells in the ventricle were observed in WT and mutant hearts at 48 hpf (Figure 7H).

Cardiomyocytes normally begin to enlarge as the zebrafish cardiac chambers emerge (36 hpf), and between 40–45 hpf more than half of ventricular cardiomyocytes increase in size^{60,61}. Thus, we asked whether cardiomyocyte size was impacted in the mutants, and could explain the decreased heart size at 48 hpf. We focused on ventricular cardiomyocytes, because in WT hearts DM-GRASP immunostaining labeled ventricular cardiomyocyte membranes, but not to any great extent atrial cells (Figure 7C). Interestingly, the size of ventricular cardiomyocytes was strikingly different between the WT and *sema3fb*^{ca305} embryos (insets, Figure 7C,D). To quantitate this change, we took confocal projections of DM-GRASP-labeled hearts and measured area, perimeter and circularity of the cardiomyocytes within the central region of the ventricle. While circularity was comparable across WT and mutant cardiomyocytes (WT 0.66 ± 0.02 , n=5 embryos (9-12 cells measured/embryo); *sema3fb*^{ca305} 0.63 ± 0.03 , n=6 embryos; Mann Whitney U, p=0.66, Figure 7I), both the area (WT $112.8 \pm 4.0 \mu\text{m}^3$, n=4 embryos; *sema3fb*^{ca305} $73.3 \pm 3.8 \mu\text{m}^3$, n=5 embryos; Mann Whitney U, p=0.016, Figure 7J) and perimeter (WT $45.6 \pm 1.8 \mu\text{m}$, n=5 embryos; *sema3fb*^{ca305} $39.2 \pm 1.0 \mu\text{m}$, n=6 embryos; Mann Whitney U, p=0.03, Figure 7K) of ventricular cardiomyocytes were reduced significantly in the mutant embryos. These data support the idea that chamber size is decreased in mutants in part because of a reduction in cardiomyocyte size.

A significant portion of the cardiac myocyte population in zebrafish arises from the integration of cardiomyocytes from the secondary heart field⁶² and cardiac neural crest (CNC)⁶³. To determine if cardiomyocytes originating from either of these two sources failed to enter the heart as a result of the loss of a known chemotropic molecule expressed therein, we assayed

markers of the secondary heart field (*latent tgfb binding protein 3a (ltbp3a)*)^{64,65} and CNC (*cysteine-rich intestinal protein 2 (crip2)*) by whole mount ISH at 48 hpf. *crip2* was expressed at similar levels in WT and mutant ventricles (Suppl Figure 5A,B), and *ltbp3a*-expressing cardiomyocytes were present in both the WT and mutant hearts (Suppl Figure 5C,D). Together, our data argue that changes in proliferation, apoptosis, and the integration of cardiomyocytes from sources external to the heart, do not contribute substantially to the small stature of the *sema3fb* mutant heart.

Because cardiomyocyte size is linked intimately to the hemodynamic forces of flow within the heart^{60,61}, we asked whether *sema3fb* mutant hearts were still smaller than their WT counterparts when cardiac flow was removed from the equation by growing hearts independent of the circulatory system. Hearts of WT and mutant embryos were explanted at 24 hpf and cultured for 24 hours. Explants were fixed and assessed by ISH for the expression of atrial *myh6* (Suppl Figure 6A,B). *myh6*⁺ mutant atria were significantly smaller (WT $2950 \pm 210 \mu\text{m}^3$, N=2, n=8 explants; *sema3fb*^{ca305} $2320 \pm 130 \mu\text{m}^3$, N=2, n=13 explants; Mann Whitney U, p=0.025) in size as compared to WT (Suppl Figure 6C), arguing that chamber defects present in the *Sema3fb* mutants independently of hemodynamic forces.

Nrp2b and Plxna3 receptors may mediate the Sema3fb signal

Sema3s signal canonically through Nrp and Plxn receptors¹³. We next asked which receptors *Sema3fb* might act through to influence cardiac chamber development by performing whole mount ISH for *plxna* and *nrp* genes in zebrafish. Expression of zebrafish *nrp* genes has been reported previously^{67,68}, and analysis of gross defects with Nrp knockdown, revealed vascular issues in *nrp1a*, *nrp1b* and *nrp2a* antisense MO-injected fish, and pericardial defects in

the Nrp2b morphants⁶⁸, pointing to a role for Nrp2b in heart development. *nrp2b* and *plxna3* mRNAs were present early alongside *sema3fb* mRNA in the bilateral heart field at 18 hpf (Suppl Figure 7A-C), with mRNAs confined to the presumptive ventricular myocardium by 24 hpf (Suppl Figure 7D; Figure 8B), as evident for *plxna3* at 48 hpf (Figure 8C). Of note, other *plxna* genes were not expressed at significant levels in heart tissue at 24 hpf (Suppl Figure 7H-L). In contrast, *sema3fb* mRNA was expressed throughout the entire myocardium (Figure 8A). To identify which heart cells express *plxna3* and *nrp2b* and can respond to *Sema3fb* we performed whole mount ISH on *Tg(flk:EGFP)* fish at 28-36 hpf. In these fish, endocardial cells in the heart are fluorescently labeled. For both *nrp2b* (Suppl Figure 7E-E'') and *plxna3* (Figure 8D-D''), ISH label was in cardiomyocytes adjacent to the EGFP-expressing endocardium. In summary, while *sema3fb* is expressed throughout the embryonic ventricle and atrial myocardium, the receptor expression data argue that *Sema3fb* signaling is likely spatially restricted to the ventricular myocardium.

To determine whether loss of function of either receptor recapitulated the *sema3fb* mutant chamber phenotypes, we performed MO-mediated knockdown of *Plxna3* and *Nrp2b* with previously characterized MOs³⁷. Knockdown of either receptor resulted in obvious edema by 48 hpf, indicating that heart function was compromised. The hearts of *nrp2b*-deficient embryos were grossly malformed, with a severe reduction in atrial size (control $10,960 \pm 320 \mu\text{m}^3$, n=14; *nrp2b* MO $5,314 \pm 670 \mu\text{m}^3$, n=10; Mann Whitney, $p < 0.0001$, Suppl Figure 7F,G, Figure 8E), suggesting a key role for Nrp2b in early cardiac development. This early role meant, however, that we could not easily assess Nrp2b function in later aspects of heart development. Thus, we restricted our analysis to the *plxna3*-deficient embryos. Importantly, similar to what we saw in *sema3fb* mutants, *plxna3* morphants (Figure 8H) exhibited significantly smaller sized atria than

control (Figure 8G) (N=2; control $11,749 \pm 420 \mu\text{m}^3$, n=13; *plxna3* MO $9,142 \pm 355 \mu\text{m}^3$, n=17; Mann Whitney U, $p < 0.0001$, Figure 8F). We next assessed heart function by live imaging of 72 hpf hearts. Similar to *sema3fb*^{ca305} embryos, *plxna3*-deficient embryos showed a reduction in heart rate (99.2 ± 1.9 beats/min, N=3, n=10; Mann Whitney, $p=0.011$, Figure 8L) as compared to controls (111.1 ± 3.1 beats/min, N=3, n=9). Yet, cardiac output (control 20.8 ± 2.9 nL/min, N=3, n=9; *plxna3* MO 14.6 ± 1.8 nL/min, N=3, n=10; Mann Whitney, $p=0.09$; Figure 8M) and ejection fraction (control $42.9 \pm 4.1\%$, N=3, n=9; *plxna3* MO $34.4 \pm 2.2\%$, N=3, n=10; Mann Whitney, $p=0.18$; Figure 8N), were not significantly different between morphant and control hearts, despite the clear heart function defect revealed by the presence of edema in the *plxna3* morphants (data not shown). Likely the partial loss-of-function that would be generated with the *plxna3* MO produced cardiac defects that were less severe than those seen in the *sema3fb* mutant, but that over time resulted in edema.

Thus, it was important to determine whether in addition to the smaller heart, the key alterations observed in chamber development present in *sema3fb* mutants were also present to some degree in the *plxna3* morphants. Importantly, the area of individual DM-GRASP immunolabeled ventricular cardiomyocytes (control $76.7 \pm 4.1 \mu\text{m}^3$, N=2, n=6 hearts; *plxna3* MO $54.6 \pm 5.2 \mu\text{m}^3$, N=2, n=11; Mann Whitney U, $p=0.019$; Figure 8O), and their perimeter (control $37.8 \pm 1.1 \mu\text{m}$, N=2, n=6; *plxna3* MO $31.9 \pm 1.3 \mu\text{m}$, N=2, n=11; Mann Whitney U, $p=0.014$; Figure 8P), were significantly smaller in morphants than controls. Circularity of the morphant cardiomyocytes was comparable to controls (control 0.68 ± 0.02 , N=2, n=6; *plxna3* MO 0.72 ± 0.02 , N=2, n=10; Mann Whitney U, $p=0.15$; Figure 8Q). Notably, the distinct border of gene expression between the ventricle and atrium was also less evident in the *plxna3* morphants, as seen by the presence of strong *tnnt2a* expression in the atria (Figure 8K) and

ectopic *bmp4a* expression in the ventricle in addition to the expected AVC labeling (Figure 8I,J,R). Together, these data argue that *Plxna3* functions downstream of *Sema3fb* in the developing cardiac system. The *plxna3* morphant heart is impacted less severely than that of the *sema3fb* mutant, presumably because of residual *Plxna3* signaling in the morphants.

Discussion

Our data argue that embryonic cardiomyocytes secrete *Sema3fb* that spatially restricts chamber-specific gene expression at the atrioventricular border to ensure proper function of the embryonic heart. We find *sema3fb* mRNA is expressed by all cardiomyocytes over the period of chamber morphogenesis and differentiation, while expression of mRNAs for the candidate receptors, *Nrp2b* and *Plxna3*, is spatially restricted to ventricular cardiomyocytes. We propose that *Sema3fb* signals inform ventricular cardiomyocytes they border cells in the atrium, which lack the machinery to sense *Sema3fb*. The consequence is the proper refinement of chamber-specific gene expression at the AVC, a feature that is disrupted in the absence of *Sema3fb*. The size of both heart chambers is smaller, we propose as a consequence of these defects in gene expression. Our work argues for the first time that a heart-derived secreted *Sema3* provides a spatially-restricted signal required for the normal refinement of the border between the ventricle and atrium, and that is required for proper chamber specific differentiation and function.

We propose a model whereby *Sema3fb* signals to *Plxna3*/*Nrp2b*-expressing ventricular cardiomyocytes in a cell autonomous manner. In support, *sema3fb* mRNA is expressed throughout the early zebrafish myocardium, while mRNAs for *Plxna3* and *Nrp2* appear restricted to the ventricle. The timing of when the heart phenotypes present in the *sema3fb* mutants argues against the causative involvement of non-cardiomyocyte tissues. In mouse, Purkinje fibers

1 secrete SEMA3A that patterns sympathetic innervation of the pacemaker node²¹. Sema3fb in
2 zebrafish likely does not play a similar role, in that the cardiomyocyte phenotypes arise prior to
3 when sympathetic nerves innervate the node⁶⁹. Epicardial cells also arrive in the heart after
4 chamber defects are evident in the 48 hpf mutants; epicardial cells emerge at around 55 hpf and
5 migrate to and attach to the myocardium by 72 hpf³³. Interestingly, Sema3fb is important in
6 zebrafish epicardial development at later stages: by 5 dpf *sema3fb* is expressed by *tbx18*+
7 epicardial cells and not cardiomyocytes, and Sema3fb regulates epicardial cell numbers in the
8 bulbus arteriosus⁷⁰. We also saw in mutant hearts the normal arrival of cardiomyocytes from
9 non-heart sources, including from the secondary heart field⁶⁴ and CNC⁷¹. One last possibility is the
10 endocardium. For instance, PLXND1 signaling within the murine endocardium promotes
11 cardiomyocytes to form trabeculae⁷². Endocardial development is delayed initially by the loss of
12 Sema3fb, however, our data argue that alterations in cardiomyocytes do not occur secondary to defects in the
13 adjacent endocardial cells. First, in both WT and *sema3fb* mutants the endocardium lines both heart
14 chambers, and the endocardium-derived heart valves form. The mutant valves do appear mis-distributed at
15 the atrioventricular border, possibly as a result of altered gene expression at the atrioventricular border, but
16 this is a mild phenotype. Second, the cardiomyocytes and not the endocardium express mRNAs for the
17 known Sema3f receptors, Plxna3 and Nrp2⁷³. Sema3fb is unlikely to act on endocardial cells via Vegfr2,
18 despite a known inhibitory effect of SEMA3F on VEGF signaling^{74,75}. SEMA3F inhibition of VEGF
19 signaling occurs via NRP and not VEGFR^{74,75}, and the endocardium does not appear to express *nrgps*⁶⁷.
20 Finally, we saw no obvious defects in heart tube assembly, a process known to be influenced by the
21 endocardium⁷⁶. These data argue that the contribution of the endocardium to the initial heart
22 impairments we observe in *sema3fb* mutants is minimal. Instead, the literature and our data support
23 strongly the idea that the cardiomyocyte defects arise from the loss of Sema3fb signaling within the

cardiomyocytes themselves. Nonetheless, it is possible that defects in valve anatomy and gene expression contribute to the cardiac dysfunction and edema seen in the *sema3fb* mutants.

Our data argue against a *Sema3fb* signal promoting directly a ventricular fate or repressing atrial/AVC fates. While both *sema3fb* and receptor mRNAs are expressed within the early bilateral heart field, ventricular and atrial cardiomyocyte progenitors are specified much earlier, prior to zebrafish gastrulation⁷⁷. Indeed, both the formation of the ventricular and atrial chambers and their subsequent morphogenesis appear to occur normally in the absence of *Sema3fb*. Furthermore, *irx1a* and *tbx5a*, transcription factors implicated in ventricular cardiomyocyte differentiation, remain expressed throughout the mutant ventricle. Thus, if *Sema3fb* has an early role in heart development, it likely acts redundantly with other mechanisms.

The main impact of *Sema3fb* loss occurs after the initial specification of the chambers. Both atrial and ventricular chambers are smaller in the absence of *Sema3fb*, and several genes and proteins whose expression normally obeys the border between the atrium and ventricle are disrupted: 1) *bmp4a* expression fails to refine to the atrioventricular band, 2) *myh7* is largely absent from the ventricular side of the border, 3) *myh6* spreads anteriorly past the atrioventricular constriction, and 4) DM-GRASP and MF20 that are normally expressed preferentially in the ventricle show significant expression throughout the atrium. These data support the idea that *Sema3fb* provides a patterned signal to ventricular cardiomyocytes at the border with the atrial chamber. The latter lacks *Plxn3*, and *Sema3fb* signaling should not be present. Likely the signal provided by *Sema3fb* does not establish the border between the chambers, as the AVC does form in the *sema3fb*^{ca305} heart. Further, several genes still exhibit the expected profound changes in expression at the border: The posterior border of the *irx1a* expression domain obeys the boundary, *bmp4a* mRNA is located preferentially at the AVC, and *myh6* mRNA is largely

restricted to the atrial chamber. Instead, we propose that *Sema3fb* allows for the sharpness of the border to emerge in a timely manner.

Ventricular cardiomyocytes in the border region can respond to *Sema3fb* while immediately adjacent atrial cardiomyocytes cannot. Our data suggest that this differential signaling ability is required for the refinement of the AVC region. In the absence of border refinement when the *Sema3fb* signal is absent, the AVC region forms, as exhibited by preferential *bmp4a* expression, but the domain's border expands into the ventricle and/or the atrium. The *Sema3f* overexpression data argues against the possibility that *Sema3f* directly regulates gene expression, as ectopic *bmp4a* expression in the 48 hpf heart is seen with both loss- and gain- of function of *Sema3f*. Rather, the overexpression data support the idea that *Sema3f* tells ventricular cardiomyocytes that they sit at the border with the atrium. If the correct spatial information is lost, either because the signal itself is gone (mutant), or the signal is abnormal with cells in the AVC region experiencing different levels of signaling (overexpression), proper refinement of the AVC region fails to occur. An imprecise AVC region leads to disrupted regulation of cardiomyocyte-specific genes in a chamber-defined manner: DM-GRASP, *myh7*, *myh6* and MF20. In support, restriction of Bmp signaling to the AVC is necessary to prevent inhibition of cardiomyocyte differentiation by *Tbx2* within the ventricular chamber^{78–80}.

Interestingly, in *sema3fb* mutants the atrium is also impacted with regard to size, functional parameters, and misexpression of ventricular and AVC markers. Given that atrial cardiomyocytes lack the machinery to respond to *Sema3fb*, altered atrial differentiation arises presumably secondarily to disrupted ventricular cardiomyocyte development. One possibility is that disrupted ventricle function impacts atrial cardiomyocytes, as the function of the two chambers shows some inter-dependence⁴⁹. Explanted mutant atria are still smaller than their WT

counterparts, however, despite having removed flow as a variable. Potentially, alterations in the secretion of factors from ventricular cardiomyocytes and/or cells of the AVC affects the differentiation of the nearby atrial cardiomyocytes. In support, both MF20 and DM-GRASP proteins appear upregulated in the atrium of *sema3fb* mutants.

The heart chambers are smaller in *sema3fb* mutants, though the data suggests that fewer cardiomyocytes is not the explanation. The expression of *fgf8a* and *tbx5a* (data not shown) in the primary heart field, and markers of secondary heart field-derived cells, are comparable in the two genotypes. Further, cell death and proliferation at 48 hpf are not impacted by *Sema3fb* loss. Indeed, similar numbers of ventricular cardiomyocytes are present in WT and mutant hearts at this stage. Instead, without *Sema3fb* these cardiomyocytes appear to be smaller than normal. The fact that the atrial chamber was smaller in the absence of changes in proliferation, apoptosis and cell integration also argues for smaller cardiomyocytes within the atria. Heart dysfunction could result in smaller cells, in that zebrafish cardiomyocytes hypertrophy normally at about 45 hpf in response to shear stresses arising from the onset of blood flow through the heart⁶¹. Yet, the atria of explanted mutant hearts were still smaller than those of WT, even though shear force was removed as a functional regulator. Potentially, *Sema3fb* signaling causes cytoskeletal rearrangements that induce ventricular cardiomyocyte expansion, but our data argue that atrial cardiomyocytes are not responding directly to *Sema3fb*. For atrial cardiomyocytes, and possibly their ventricular counterparts, disrupted expression of differentiation genes remains a potential explanation for the failure of cardiomyocytes to undergo hypertrophy.

The multigenic and heterogenous disease Hypoplastic Left Heart Syndrome (HLHS) is characterized by a hypoplastic left ventricle, aorta and mitral valve, and without treatment, is generally fatal^{81,82}. The Ohia mouse harbours mutations in *SAP130* and *PCDH9A*, and presents

with hypoplasia of the left ventricle, likely due to an intrinsic deficit in cardiomyocyte proliferation and differentiation⁸³. Our data support the idea of defective differentiation of ventricular cardiomyocytes as a potential contributor to HLHS. Different from what is often observed in HLHS, however, the ventricular defect in the *sema3fb* mutants translates to a smaller atrium. This involvement of the atrium may reflect a critical interdependence of the atrium and ventricle in the two-chambered zebrafish heart. While *sema3fb* mRNA in the ventricle is largely downregulated by 72 hpf (data not shown), earlier defects in differentiation generates ventricular cardiomyocytes that fail to maintain normal cardiac function as the fish mature. The fact that the edema in these fish appears to resolve, and the mutant alleles are viable, argues for mechanisms that ultimately compensate for the loss of *Sema3fb*.

Here we provide evidence for a secreted *Sema* functioning as an extrinsic regulator of regional differentiation in the cardiovascular system. *Sema3s* are known to be important for development of the mouse cardiovascular system¹⁶. In some cases, the roles are myocardium-independent, as is the case for *SEMA3A* and innervation of the heart²¹, and *Sema3d* and *SEMA3C* in CNC-dependent generation of cardiomyocytes^{84,85}. Here, we suggest a novel cardiomyocyte autonomous function of *Sema3fb* in governing the molecular differentiation of cardiomyocytes. While *Sema3f* is expressed in the cardiac cushions and in scattered cells throughout the myocardium of the embryonic chick heart⁸⁶, no cardiac phenotype is described in the *Sema3f* mouse mutant⁸⁷, nor is *Nrp2* expressed by murine cardiac muscle⁸⁸. In mouse, the transmembrane *SEMA6D* promotes cardiomyocyte proliferation⁸⁹. In concert with our findings, these data support the idea that *Semas* directly regulate cardiomyocyte development. Additional roles for *Semas* will likely be identified in heart development as more subtle cardiovascular defects are assessed in loss-of-function models.

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References:

1. van der Linde D, Konings EEM, Slager MA, Witsenburg M, Helbing WA, Takkenberg JJM, Roos-Hesselink JW. Birth prevalence of congenital heart disease worldwide: a systematic review and meta-analysis. *J Am Coll Cardiol*. 2011;58:2241–2247.
2. DeRuiter MC, Poelmann RE, VanderPlas-de Vries I, Mentink MM, Gittenberger-de Groot AC. The development of the myocardium and endocardium in mouse embryos. Fusion of two heart tubes? *Anat Embryol (Berl)*. 1992;185:461–473.
3. Chien KR, Zhu H, Knowlton KU, Miller-Hance W, van-Bilsen M, O'Brien TX, Evans SM. Transcriptional regulation during cardiac growth and development. *Annu Rev Physiol*. 1993;55:77–95.
4. Lyons GE, Schiaffino S, Sassoon D, Barton P, Buckingham M. Developmental regulation of myosin gene expression in mouse cardiac muscle. *J Cell Biol*. 1990;111:2427–2436.
5. Battle E, Wilkinson DG. Molecular mechanisms of cell segregation and boundary formation in development and tumorigenesis. *Cold Spring Harb Perspect Biol*. 2012;4:a008227.
6. Addison M, Wilkinson DG. Segment Identity and Cell Segregation in the Vertebrate Hindbrain. *Curr Top Dev Biol*. 2016;117:581–596.
7. Bruneau BG, Logan M, Davis N, Levi T, Tabin CJ, Seidman JG, Seidman CE. Chamber-specific cardiac expression of Tbx5 and heart defects in Holt-Oram syndrome. *Dev Biol*. 1999;211:100–108.
8. Garside VC, Chang AC, Karsan A, Hoodless PA. Co-ordinating Notch, BMP, and TGF- β signaling during heart valve development. *Cell Mol Life Sci CMLS*. 2013;70:2899–2917.
9. Marques SR, Lee Y, Poss KD, Yelon D. Reiterative roles for FGF signaling in the establishment of size and proportion of the zebrafish heart. *Dev Biol*. 2008;321:397–406.
10. Bao ZZ, Bruneau BG, Seidman JG, Seidman CE, Cepko CL. Regulation of chamber-specific gene expression in the developing heart by Irx4. *Science*. 1999;283:1161–1164.
11. Wang J, Klysik E, Sood S, Johnson RL, Wehrens XHT, Martin JF. Pitx2 prevents susceptibility to atrial arrhythmias by inhibiting left-sided pacemaker specification. *Proc Natl Acad Sci U S A*. 2010;107:9753–9758.
12. Alto LT, Terman JR. Semaphorins and their Signaling Mechanisms. *Methods Mol Biol Clifton NJ*. 2017;1493:1–25.
13. Fujisawa H. Discovery of semaphorin receptors, neuropilin and plexin, and their functions in neural development. *J Neurobiol*. 2004;59:24–33.
14. Jongbloets BC, Pasterkamp RJ. Semaphorin signalling during development. *Dev Camb Engl*. 2014;141:3292–3297.
15. Mecollari V, Nieuwenhuis B, Verhaagen J. A perspective on the role of class III semaphorin signaling in central nervous system trauma. *Front Cell Neurosci*. 2014;8. doi:10.3389/fncel.2014.00328.

16. Epstein JA, Aghajanian H, Singh MK. Semaphorin Signaling in Cardiovascular Development. *Cell Metab.* 2015;21:163–173.
17. Feiner L, Webber AL, Brown CB, Lu MM, Jia L, Feinstein P, Mombaerts P, Epstein JA, Raper JA. Targeted disruption of semaphorin 3C leads to persistent truncus arteriosus and aortic arch interruption. *Dev Camb Engl.* 2001;128:3061–3070.
18. Degenhardt K, Singh MK, Aghajanian H, Massera D, Wang Q, Li J, Li L, Choi C, Yzaguirre AD, Francey LJ, Gallant E, Krantz ID, Gruber PJ, Epstein JA. Semaphorin 3d signaling defects are associated with anomalous pulmonary venous connections. *Nat Med.* 2013;19:760–765.
19. Gu C, Yoshida Y, Livet J, Reimert DV, Mann F, Merte J, Henderson CE, Jessell TM, Kolodkin AL, Ginty DD. Semaphorin 3E and plexin-D1 control vascular pattern independently of neuropilins. *Science.* 2005;307:265–268.
20. Kutschera S, Weber H, Weick A, De Smet F, Genove G, Takemoto M, Prahst C, Riedel M, Mikelis C, Baulande S, Champseix C, Kummerer P, Conseiller E, Multon M-C, Heroult M, Bicknell R, Carmeliet P, Betsholtz C, Augustin HG. Differential endothelial transcriptomics identifies semaphorin 3G as a vascular class 3 semaphorin. *Arterioscler Thromb Vasc Biol.* 2011;31:151–159.
21. Ieda M, Kanazawa H, Kimura K, Hattori F, Ieda Y, Taniguchi M, Lee J-K, Matsumura K, Tomita Y, Miyoshi S, Shimoda K, Makino S, Sano M, Kodama I, Ogawa S, Fukuda K. Sema3a maintains normal heart rhythm through sympathetic innervation patterning. *Nat Med.* 2007;13:604–612.
22. Pelster B, Burggren WW. Disruption of hemoglobin oxygen transport does not impact oxygen-dependent physiological processes in developing embryos of zebra fish (*Danio rerio*). *Circ Res.* 1996;79:358–362.
23. Fishman MC, Stainier DY, Breitbart RE, Westerfield M. Zebrafish: genetic and embryological methods in a transparent vertebrate embryo. *Methods Cell Biol.* 1997;52:67–82.
24. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn Off Publ Am Assoc Anat.* 1995;203:253–310.
25. Proulx K, Lu A, Sumanas S. Cranial vasculature in zebrafish forms by angioblast cluster-derived angiogenesis. *Dev Biol.* 2010;348:34–46.
26. Montague TG, Cruz JM, Gagnon JA, Church GM, Valen E. CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. *Nucleic Acids Res.* 2014;42:W401–7.
27. Gagnon JA, Valen E, Thyme SB, Huang P, Ahkmetova L, Pauli A, Montague TG, Zimmerman S, Richter C, Schier AF. Efficient mutagenesis by Cas9 protein-mediated oligonucleotide insertion and large-scale assessment of single-guide RNAs. *PloS One.* 2014;9:e98186.
28. Meeker ND, Hutchinson SA, Ho L, Trede NS. Method for isolation of PCR-ready genomic DNA from zebrafish tissues. *BioTechniques.* 2007;43:610, 612, 614.
29. Peterson SM, Freeman JL. RNA Isolation from Embryonic Zebrafish and cDNA Synthesis for Gene Expression Analysis. *J Vis Exp JoVE.* 2009. doi:10.3791/1470.

30. Thisse C, Thisse B. High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat Protoc.* 2008;3:59–69.
31. Hoage T, Ding Y, Xu X. Quantifying Cardiac Functions in Embryonic and Adult Zebrafish. In: Peng X, Antonyak M, eds. *Cardiovascular Development: Methods and Protocols*. Totowa, NJ: Humana Press; 2012: 11–20.
32. Sullivan-Brown J, Bisher ME, Burdine RD. Embedding, serial sectioning and staining of zebrafish embryos using JB-4 resin. *Nat Protoc.* 2011;6:46–55.
33. Peralta M, González-Rosa JM, Marques IJ, Mercader N. The Epicardium in the Embryonic and Adult Zebrafish. *J Dev Biol.* 2014;2:101–116.
34. Kwan KM, Fujimoto E, Grabher C, Mangum BD, Hardy ME, Campbell DS, Parant JM, Yost HJ, Kanki JP, Chien C-B. The Tol2kit: a multisite gateway-based construction kit for Tol2 transposon transgenesis constructs. *Dev Dyn Off Publ Am Assoc Anat.* 2007;236:3088–3099.
35. Huang C-J, Tu C-T, Hsiao C-D, Hsieh F-J, Tsai H-J. Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. *Dev Dyn Off Publ Am Assoc Anat.* 2003;228:30–40.
36. Phng L-K, Gebala V, Bentley K, Philippides A, Wacker A, Mathivet T, Sauter L, Stanchi F, Belting H-G, Affolter M, Gerhardt H. Formin-mediated actin polymerization at endothelial junctions is required for vessel lumen formation and stabilization. *Dev Cell.* 2015;32:123–132.
37. Tanaka H, Maeda R, Shoji W, Wada H, Masai I, Shiraki T, Kobayashi M, Nakayama R, Okamoto H. Novel mutations affecting axon guidance in zebrafish and a role for plexin signalling in the guidance of trigeminal and facial nerve axons. *Dev Camb Engl.* 2007;134:3259–3269.
38. Stainier DY, Lee RK, Fishman MC. Cardiovascular development in the zebrafish. I. Myocardial fate map and heart tube formation. *Development.* 1993;119:31–40.
39. Fishman MC, Chien KR. Fashioning the vertebrate heart: earliest embryonic decisions. *Dev Camb Engl.* 1997;124:2099–2117.
40. Yalcin HC, Amindari A, Butcher JT, Althani A, Yacoub M. Heart function and hemodynamic analysis for zebrafish embryos. *Dev Dyn.* 2017;246:868–880.
41. Beis D, Bartman T, Jin S-W, Scott IC, D’Amico LA, Ober EA, Verkade H, Frantsve J, Field HA, Wehman A, Baier H, Tallafuss A, Bally-Cuif L, Chen J-N, Stainier DYR, Jungblut B. Genetic and cellular analyses of zebrafish atrioventricular cushion and valve development. *Development.* 2005;132:4193–4204.
42. Sehnert AJ, Huq A, Weinstein BM, Walker C, Fishman M, Stainier DYR. Cardiac troponin T is essential in sarcomere assembly and cardiac contractility. *Nat Genet.* 2002;31:106–110.
43. Pawlak M, Kedzierska KZ, Migdal M, Nahia KA, Ramilowski JA, Bugajski L, Hashimoto K, Marconi A, Piwocka K, Carninci P, Winata CL. Dynamics of cardiomyocyte transcriptome and chromatin landscape demarcates key events of heart development. *Genome Res.* 2019;29:506–519.

44. Tamagnone L, Artigiani S, Chen H, He Z, Ming GI, Song H, Chedotal A, Winberg ML, Goodman CS, Poo M, Tessier-Lavigne M, Comoglio PM. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell*. 1999;99:71–80.
45. Denvir MA, Tucker CS, Mullins JJ. Systolic and diastolic ventricular function in zebrafish embryos: Influence of norepinephrine, MS-222 and temperature. *BMC Biotechnol*. 2008;8:21.
46. Miura GI, Yelon D. A guide to analysis of cardiac phenotypes in the zebrafish embryo. *Methods Cell Biol*. 2011;101:161–180.
47. Reifers F, Walsh EC, Léger S, Stainier DY, Brand M. Induction and differentiation of the zebrafish heart requires fibroblast growth factor 8 (fgf8/acerebellar). *Dev Camb Engl*. 2000;127:225–235.
48. Yelon D, Horne SA, Stainier DY. Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. *Dev Biol*. 1999;214:23–37.
49. Berdoudo E, Coleman H, Lee DH, Stainier DYR, Yelon D. Mutation of weak atrium/atrial myosin heavy chain disrupts atrial function and influences ventricular morphogenesis in zebrafish. *Development*. 2003;130:6121–6129.
50. Brown DR, Samsa LA, Qian L, Liu J. Advances in the Study of Heart Development and Disease Using Zebrafish. *J Cardiovasc Dev Dis*. 2016;3. doi:10.3390/jcdd3020013.
51. Grant MG, Patterson VL, Grimes DT, Burdine RD. Modeling Syndromic Congenital Heart Defects in Zebrafish. In: *Current Topics in Developmental Biology*. Academic Press Inc.; 2017: 1–40.
52. Scherz PJ, Huiskens J, Sahai-Hernandez P, Stainier DYR. High-speed imaging of developing heart valves reveals interplay of morphogenesis and function. *Development*. 2008;135:1179–1187.
53. Bader D, Masaki T, Fischman DA. Immunochemical analysis of myosin heavy chain during avian myogenesis in vivo and in vitro. *J Cell Biol*. 1982;95:763–770.
54. de la Pompa JL, Timmerman LA, Takimoto H, Yoshida H, Elia AJ, Samper E, Potter J, Wakeham A, Marengere L, Langille BL, Crabtree GR, Mak TW. Role of the NF-ATc transcription factor in morphogenesis of cardiac valves and septum. *Nature*. 1998;392:182–186.
55. Wong KS, Rehn K, Palencia-Desai S, Kohli V, Hunter W, Uhl JD, Rost MS, Sumanas S. Hedgehog signaling is required for differentiation of endocardial progenitors in zebrafish. *Dev Biol*. 2012;361:377–391.
56. Cheng CW, Hui C, Strähle U, Cheng SH. Identification and expression of zebrafish Iroquois homeobox gene *irx1*. *Dev Genes Evol*. 2001;211:442–444.
57. Garrity DM, Childs S, Fishman MC. The heartstrings mutation in zebrafish causes heart/fin Tbx5 deficiency syndrome. *Development*. 2002;129:4635–4645.
58. Walsh EC, Stainier DYR. UDP-Glucose Dehydrogenase Required for Cardiac Valve Formation in Zebrafish. *Science*. 2001;293:1670–1673.
59. Plant T, Eamsamrongs S, Sanchez-Garcia MA, Reyes L, Renshaw SA, Coelho P, Mirchandani AS, Morgan J-M, Ellett FE, Morrison T, Humphries D, Watts ER, Murphy F, Raffo-Iraolagoitia XL,

- 1 Zhang A, Cash JL, Loynes C, Elks PM, Van Eeden F, Carlin LM, Furley AJ, Whyte MK, Walmsley
2 SR. Semaphorin 3F signaling actively retains neutrophils at sites of inflammation. *J Clin Invest*.
3 2020;130:3221–3237.
- 4 60. Lin Y-F, Swinburne I, Yelon D. Multiple influences of blood flow on cardiomyocyte hypertrophy in
5 the embryonic zebrafish heart. *Dev Biol*. 2012;362:242–253.
- 6 61. Auman HJ, Coleman H, Riley HE, Olale F, Tsai H-J, Yelon D. Functional Modulation of Cardiac
7 Form through Regionally Confined Cell Shape Changes. *PLOS Biol*. 2007;5:e53.
- 8 62. de Pater E, Clijsters L, Marques SR, Lin Y-F, Garavito-Aguilar ZV, Yelon D, Bakkers J. Distinct
9 phases of cardiomyocyte differentiation regulate growth of the zebrafish heart. *Dev Camb Engl*.
10 2009;136:1633–1641.
- 11 63. Sato M, Yost HJ. Cardiac neural crest contributes to cardiomyogenesis in zebrafish. *Dev Biol*.
12 2003;257:127–139.
- 13 64. Guner-Ataman B, Paffett-Lugassy N, Adams MS, Nevis KR, Jahangiri L, Obregon P, Kikuchi K,
14 Poss KD, Burns CE, Burns CG. Zebrafish second heart field development relies on progenitor
15 specification in anterior lateral plate mesoderm and nkx2.5 function. *Development*. 2013;140:1353–
16 1363.
- 17 65. Zhou Y, Cashman TJ, Nevis KR, Obregon P, Carney SA, Liu Y, Gu A, Mosimann C, Sondalle S,
18 Peterson RE, Heideman W, Burns CE, Burns CG. Latent TGF- β binding protein 3 identifies a second
19 heart field in zebrafish. *Nature*. 2011;474:645–648.
- 20 66. Sun X, Zhang R, Lin X, Xu X. Wnt3a regulates the development of cardiac neural crest cells by
21 modulating expression of cysteine-rich intestinal protein 2 in rhombomere 6. *Circ Res*.
22 2008;102:831–839.
- 23 67. Bovenkamp DE, Goishi K, Bahary N, Davidson AJ, Zhou Y, Becker T, Becker CG, Zon LI,
24 Klagsbrun M. Expression and mapping of duplicate neuropilin-1 and neuropilin-2 genes in
25 developing zebrafish. *Gene Expr Patterns GEP*. 2004;4:361–370.
- 26 68. Martyn U, Schulte-Merker S. Zebrafish neuropilins are differentially expressed and interact with
27 vascular endothelial growth factor during embryonic vascular development. *Dev Dyn Off Publ Am*
28 *Assoc Anat*. 2004;231:33–42.
- 29 69. Burczyk MS, Burkhalter MD, Tena TC, Grisanti LA, Kauk M, Matysik S, Donow C, Kustermann M,
30 Rothe M, Cui Y, Raad F, Laue S, Moretti A, Zimmermann W-H, Wess J, Kühl M, Hoffmann C,
31 Tilley DG, Philipp M. Muscarinic receptors promote pacemaker fate at the expense of secondary
32 conduction system tissue in zebrafish. *JCI Insight*. 2019;4. doi:10.1172/jci.insight.121971.
- 33 70. Weinberger M, Simões FC, Patient R, Sauka-Spengler T, Riley PR. Functional Heterogeneity within
34 the Developing Zebrafish Epicardium. *Dev Cell*. 2020. doi:10.1016/j.devcel.2020.01.023.
- 35 71. Cavanaugh AM, Huang J, Chen J-N. Two developmentally distinct populations of neural crest cells
36 contribute to the zebrafish heart. *Dev Biol*. 2015;404:103–112.

72. Sandireddy R, Cibi DM, Gupta P, Singh A, Tee N, Uemura A, Epstein JA, Singh MK. Semaphorin 3E/PlexinD1 signaling is required for cardiac ventricular compaction. *JCI Insight*. 2019;4. doi:10.1172/jci.insight.125908.
73. Sharma A, Verhaagen J, Harvey AR. Receptor complexes for each of the Class 3 Semaphorins. *Front Cell Neurosci*. 2012;6. doi:10.3389/fncel.2012.00028.
74. Parker MW, Xu P, Guo H-F, Vander Kooi CW. Mechanism of selective VEGF-A binding by neuropilin-1 reveals a basis for specific ligand inhibition. *PloS One*. 2012;7:e49177.
75. Guo H-F, Li X, Parker MW, Waltenberger J, Becker PM, Vander Kooi CW. Mechanistic basis for the potent anti-angiogenic activity of semaphorin 3F. *Biochemistry*. 2013;52:7551–7558.
76. Holtzman NG, Schoenebeck JJ, Tsai H-J, Yelon D. Endocardium is necessary for cardiomyocyte movement during heart tube assembly. *Dev Camb Engl*. 2007;134:2379–2386.
77. Staudt D, Stainier D. Uncovering the molecular and cellular mechanisms of heart development using the zebrafish. *Annu Rev Genet*. 2012;46:397–418.
78. Singh R, Horsthuis T, Farin HF, Grieskamp T, Norden J, Petry M, Wakker V, Moorman AFM, Christoffels VM, Kispert A. Tbx20 interacts with smads to confine tbx2 expression to the atrioventricular canal. *Circ Res*. 2009;105:442–452.
79. Yamada M, Revelli JP, Eichele G, Barron M, Schwartz RJ. Expression of chick Tbx-2, Tbx-3, and Tbx-5 genes during early heart development: evidence for BMP2 induction of Tbx2. *Dev Biol*. 2000;228:95–105.
80. Habets PEMH, Moorman AFM, Clout DEW, van Roon MA, Lingbeek M, van Lohuizen M, Campione M, Christoffels VM. Cooperative action of Tbx2 and Nkx2.5 inhibits ANF expression in the atrioventricular canal: implications for cardiac chamber formation. *Genes Dev*. 2002;16:1234–1246.
81. Delmo Walter EMB, Hübner M, Alexi-Meskishvili V, Miera O, Weng Y, Loforte A, Berger F, Hetzer R. Staged surgical palliation in hypoplastic left heart syndrome and its variants. *J Card Surg*. 2009;24:383–391.
82. Gordon BM, Rodriguez S, Lee M, Chang R-K. Decreasing Number of Deaths of Infants with Hypoplastic Left Heart Syndrome. *J Pediatr*. 2008;153:354–358.
83. Liu X, Yagi H, Saeed S, Bais AS, Gabriel GC, Chen Z, Peterson KA, Li Y, Schwartz MC, Reynolds WT, Saydmohammed M, Gibbs B, Wu Y, Devine W, Chatterjee B, Klena NT, Kostka D, de Mesy Bentley KL, Ganapathiraju MK, Dexheimer P, Leatherbury L, Khalifa O, Bhagat A, Zahid M, Pu W, Watkins S, Grossfeld P, Murray SA, Porter GA, Tsang M, Martin LJ, Benson DW, Aronow BJ, Lo CW. The complex genetics of hypoplastic left heart syndrome. *Nat Genet*. 2017;49:1152–1159.
84. Sato M, Tsai HJ, Yost HJ. Semaphorin3D regulates invasion of cardiac neural crest cells into the primary heart field. *Dev Biol*. 2006;298:12–21.
85. Plein A, Calmont A, Fantin A, Denti L, Anderson NA, Scambler PJ, Ruhrberg C. Neural crest-derived SEMA3C activates endothelial NRP1 for cardiac outflow tract septation. *J Clin Invest*. 2015;125:2661–2676.

86. Jin Z, Chau MD, Bao Z-Z. Sema3D, Sema3F, and Sema5A Are Expressed in Overlapping and Distinct Patterns in Chick Embryonic Heart. *Dev Dyn Off Publ Am Assoc Anat.* 2006;235:163–169.
87. Sahay A, Molliver ME, Ginty DD, Kolodkin AL. Semaphorin 3F is critical for development of limbic system circuitry and is required in neurons for selective CNS axon guidance events. *J Neurosci Off J Soc Neurosci.* 2003;23:6671–6680.
88. Bielenberg DR, Seth A, Shimizu A, Pelton K, Cristofaro V, Ramachandran A, Zwaans BMM, Chen C, Krishnan R, Seth M, Huang L, Takashima S, Klagsbrun M, Sullivan MP, Adam RM. Increased smooth muscle contractility in mice deficient for neuropilin 2. *Am J Pathol.* 2012;181:548–559.
89. Sun Q, Peng Y, Zhao Q, Yan S, Liu S, Yang Q, Liu K, Rokosh DG, Jiao K. SEMA6D regulates perinatal cardiomyocyte proliferation and maturation in mice. *Dev Biol.* 2019;452:1–7.

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4 **Figure 1: *sema3fb* is expressed by cardiomyocytes of the developing heart. A-F) Whole**

5 mount ISH of 28 hpf (A,B) and 48 hpf (F) zebrafish hearts labeled with antisense riboprobes

6 for *sema3fb* (A,F) and the cardiomyocyte marker *tnnt2a* (B). Transverse section through the

7 heart at 36 hpf reveals *sema3fb* ISH signal present in both chambers of the heart (C) in

cardiomyocytes (asterisk) and not the endocardium as labeled by GFP (D) in a *Tg(flk:EGFP)* background. Blend of C and D shown in E. **G)** RT-PCR analysis indicates that exon 2 is skipped in the *sema3fb*^{ca305} allele; no PCR product is generated with a forward primer in exon 2, but PCR product is generated with an exon 3-exon 4 primer combination. In contrast, exon 2 is present in the *sema3fb*^{ca306} allele, as demonstrated by a PCR product generated with an exon 2-exon 3 primer combination. **H)** Chromosomal overview of the *sema3fb* locus targeted by CRISPR/Cas9 mutagenesis to exon 2 (guide RNA: B1) and primers used to identify the mutation. *sema3fb*^{ca305} mutants have a 19 bp deletion (dots) and *sema3fb*^{ca306} mutants have a 10 bp insertion (red sequence). Schematic representation of WT and mutant proteins. In the *sema3fb*^{ca305} allele, exon 1 is spliced in frame with exon 3, but, with the loss of exon 2, is missing 62 amino acids (aa) at the beginning of a protein that would be translated; sequence analysis indicates that both the signal sequence (ss) and the first 14 aa of the SEMA domain are missing. In the *sema3fb*^{ca306} allele, the 10 bp insertion introduces a frame shift and a premature stop codon to generate a predicted truncated 58 aa protein. F, forward primer; R, reverse primer; UTR, untranslated region. A, anterior; a, atrium; e, eye; h, heart; P, posterior; v, ventricle. Scale bar: 50 μ m.

Figure 2. *sema3fb* mutants have cardiac edema. **A)** Representative brightfield images of 72 hpf *sema3fb*^{+/+}, *sema3fb*^{ca305} and *sema3fb*^{ca306} embryos. Dotted line outlines the heart region. **B)** The percent of embryos of the different genotypes that present with cardiac edema at 48 hpf (N=3, n=60 for each genotype) and 72 hpf (N=3, n=60 for each genotype). **C)** Representative brightfield images showing the severe mutant phenotype that displays major cardiac edema and failure to thrive (C), and the abnormal linear arrangement of the heart (C', inset in C). **D)**

1 Quantitation of the edema phenotype at 72 hpf (N=3, n=180 per genotype). **E)** Quantitation
2 reveals that the average ventricle wall width (μm) in *sema3fb^{ca305}* embryos is significantly
3 thinner (*p=0.024) than that of WT hearts. Error bars represent SEM. Statistics represent the
4 Mann-Whitney U test. Scale bar in A and C is 1 mm.
5

Figure 3. *sema3fb* mutant embryos have reduced cardiac function. A-B) Still images from videos of beating WT and *sema3fb*^{ca305} mutant hearts at 72 hpf. Ventricle measurements were made as indicated, in both the long axis (white line, L: length) and short axis (black line, W: width) to determine ventricle volumes. ba: bulbus arteriosus. C-D) Quantitation of the average ventricle volume at end diastole (C) and end systole (D) reveals a significantly decreased capacity in the *sema3fb* mutant (**p=0.0027 and **p=0.0063, respectively) as compared to WT. E-I) Graphs showing average heart rate (***p=0.0008) (E), cardiac output (*p=0.026) (F), stroke volume (*p=0.033) (G), ejection fraction (p=0.45) (H), and fractional shortening

1 (p=0.17) (I). Error bars are SEM over 3 independent experiments (n=12 *sema3fb*^{+/+}, n=20
 2 *sema3fb*^{ca305}). Statistics represent the Mann-Whitney U test. Scale Bar: 100 μm.

3

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Figure 4. Disrupted heart valve development with the loss of Sema3fb. A,B) Histological sections stained by hematoxylin and eosin showing the ventricle walls (yellow brackets) and valves (arrowheads within inset) of WT (A, A') and *sema3fb*^{ca305} embryos (B, B') at 72 hpf. C-D) Physical presence of the AV valve was confirmed in confocal projections of 72 hpf hearts immunolabeled by the myocardial marker MF20 (grey) on an endocardial-labeled transgenic line *Tg(kdrl:mCherry)* (arrowheads C', D') of WT (C, C') and *sema3fb*^{ca305} embryos (D, D'). E-

1 **F)** Labeling in WT (E) and *sema3fb*^{ca305} (F) embryos at 28 hpf reveals a smaller endocardial
2 domain, as marked by *nfat1c* ISH label (yellow bars) in the mutant. **G-H)** By 48 hpf, the
3 endocardial marker *nfat1c* is concentrated at the AVC (arrowheads) in WT (G) and mutant (H)
4 fish. **I)** The endocardial marker *vegfr2* is expressed in the atrium and ventricle of both 48 hpf
5 WT and *sema3fb*^{ca305} fish. **J-L)** Quantitation in WT and mutant fish of the: (1) length of the AV
6 valve, as marked by mCherry in the *Tg(kdrl:mCherry)* background (J; N=2, n=10 *sema3fb*^{+/+},
7 n=12 *sema3fb*^{ca305}), (2) the ratio of the length of the valve present in the atrium to that
8 measured in the ventricle (K; N=2, n=10 *sema3fb*^{+/+}, n=11 *sema3fb*^{ca305}), and (3) the length of
9 the *nfat1c* domain (marked by yellow bars in E and F) in 28 hpf WT and *sema3fb*^{ca305} embryos
10 (L; N=2, n=13 *sema3fb*^{+/+}, n=15 *sema3fb*^{ca305}). Error bars are SEM. Statistics represent the
11 Mann-Whitney U test (***; p=0.0003 in K; p<0.001 in L).

1 present with smaller *myh7* positive ventricles (N=2, 17/20 *sema3fb*^{ca305}; N=2, 15/18
2 *sema3fb*^{ca306}) than WT (N=2, n=19/20) (E). Arrows indicate ectopic *myh7* label in the atria of
3 *sema3fb* mutants. **H)** Quantitation (purple line, schematic) reveals significantly shorter ventricles
4 in *sema3fb*^{ca305} (N=3, n=27, *p=0.009) and *sema3fb*^{ca306} (N=3, n=26, *p=0.013) as compared to
5 WT (N=3, n=28) embryos. **I-K)** Both *sema3fb* mutant alleles present with observably smaller
6 *myh6* positive atria (N=2, 18/21 *sema3fb*^{ca305}; N=2, 15/17 *sema3fb*^{ca306}) (J-K) than in WT (N=2,
7 n=19/19) (I). Asterisks indicate ectopic *myh6* label in the ventricles of *sema3fb* mutants. **L)**
8 Quantitation of surface area (purple fill) reveals significantly smaller atria in *sema3fb*^{ca305} (N=3,
9 n=19, ***p=0.0001) and *sema3fb*^{ca306} (N=2, n=7, ***p<0.0001) hearts as compared to WT
10 (N=3, n=19). (Kruskal Wallis One Way ANOVA, Dunn's multiple comparisons test) **M-P)** *myh6*
11 expression at 36 hpf (M,N), revealing expression anterior to the atrioventricular constriction
12 (yellow arrowheads), and at 72 hpf (O,P). **Q)** Mean *myh7* negative area measured between the
13 atrioventricular constriction and the posterior border of high *myh7* expression (N=3; WT n=28,
14 *sema3fb*^{ca305} n=26, *sema3fb*^{ca306} n=18; p<0.0001, Kruskal Wallis One Way ANOVA, Dunn's
15 multiple comparisons test). **R)** Mean area of *myh6* ISH label at 72 hpf (N=2; WT n=
16 *sema3fb*^{ca305}; p=0.0021, Mann-Whitney U test). A: anterior; P: posterior. Scale bar: 50 μ m.

Figure 6. Chamber identity disrupted at the AVC with loss and overexpression of Sema3f.

Ventral images of 48 hpf (A-G) whole mount *sema3f*^{+/+} and *sema3f*^{ca305} embryos processed for ISH. **A)** The cardiomyocyte *tnnt2a* marker does not show the sharp expression change at the border between the atrium and ventricle in the *sema3f*^{ca305} mutant heart that is present in WT (white arrowheads). **B-E)** Expression of the ventricle markers *irx1a* (B-C; N=3; WT n=15/15; *sema3f*^{ca305} n=16/16) and *tbx5a* (D-E; N=1; WT n=9/9; *sema3f*^{ca305} n=7/7) are unchanged in *sema3f*^{ca305} as compared to WT hearts. **F-I)** Expression of *bmp4a* is restricted to the AV valves (white arrow) in WT hearts at 48 hpf (F, N=4, 70%, n=21/30) and 72 hpf (H, N=1, n=8/9), but expands (white arrowheads) into the ventricle and/or atria of mutants (G, 48 hpf, 80%, n=36/45 with expansion; I, 72 hpf, n=10/11). **J)** RT-qPCR reveals significant upregulation of *bmp4a* (*p=0.036, N=5) and no change in *tbx5a* (p=0.7, N=3) mRNA levels in *sema3f*^{ca305} mutant vs. WT embryos. Error bars represent standard deviation (SD), and the statistics represent the

Mann-Whitney U test. **K)** Graph of the mean area of the *bmp4a* domain measured from images of whole mount *bmp4a* ISH (N=4, WT n=30, mutant n=45; Mann-Whitney U test, p<0.0001). **L-O)** Ventral images of 48 hpf whole mount embryos injected at the one cell stage with *myl7:sema3f* plasmid to drive Sema3f expression in heart cardiomyocytes, and processed by immunolabeling for myc to visualize the myc-tagged Sema3f (L), and by ISH for *bmp4a* (M). With Sema3f overexpression *bmp4a* expands into the ventricle and/or atria (white arrowheads, J; N=2, n=24/37 expanded) as compared to control (*bmp4a* n=17/20 normal). **O)** Graph of the mean area of the *bmp4a* domain measured from whole mount ISH mages (N=2, control n=20, Sema3f overexpression n=36; Mann-Whitney U test, p<0.001). Scale bar in A (panels A-C) and B (panels B-I, L-M) is 50 μ m.

Figure 7. Loss of Sema3fb disrupts the normal pattern of ventricular cardiomyocyte

protein expression. A-D) Confocal images of 48 hpf whole mount hearts processed by

immunohistochemistry for pHH3 and MF20 (A,B), and DM-GRASP (C,D). MF20 and DM-

GRASP are more highly expressed in the WT ventricle, with protein expression exhibiting a

fairly sharp border at the atrioventricular constriction (yellow arrowheads) (A, N=1, n=5/5, and

C, N=3, n=11/12). Expression of both proteins expands into the atrium of the mutant hearts (B,

N=1, n=8/9 and D (white arrowhead), N=3, n=10/12). **E)** Average RGB immunolabel intensity

for MF-20 across the antero-posterior axis of the heart reveals that WT (n=5) hearts exhibit

high expression in the ventricle that sharply tapers at the border with the atrium (~150 arbitrary

units (a.u.); arrowhead), while the intensity profile in *Sema3fb*^{ca305} (n=9) mutants remains stable

through both chambers. Note that hearts are smaller in the *sema3fb* mutants. **F)** Mean number of actively dividing MF20+ cardiomyocytes as revealed by pHH3 immunostaining (WT n=16 hearts, mutant n=27, p=0.71). **G)** Quantitation of the number of TUNEL positive MF20-expressing cardiomyocytes at 48 hpf (N=2, n=8, both genotypes, p=0.99). **H)** Mean number of MF20+ ventricular cardiomyocytes in DAPI-labelled hearts (n=10 WT and n=7 mutants; p=0.72). **I-K)** Mean ventricular cardiomyocyte area (J) and perimeter (K) are significantly smaller in *sema3fb*^{ca305} as compared to WT (p=0.016 and p=0.03, respectively), while circularity (I) is not impacted (p=0.66). Measurements were made using N=3, n=4-5 WT and N=3, n=6 mutant embryos, with 9-12 DM-GRASP labelled cells measured per embryo. Error bars represent SEM. Statistics represent the Mann-Whitney U test. A: anterior; P: posterior. Scale bar: 50 μ m.

view of *plxna3* mRNA in the ventricle but not atrium at 48 hpf (C). **D)** Sagittal section of *plxna3* (D') ISH label at 36 hpf on a *Tg(flk:EGFP)* background to label the GFP+ endocardium (D). The blend of these two labels is shown in D''. e, eye; h, heart; Hb, hindbrain; pv, presumptive ventricle; Tec, optic tectum. **E-F)** Mean atrial area is reduced significantly in *nrp2b* deficient embryos ($p < 0.0001$, $n = 10$ embryos; E) as compared to controls ($n = 14$), and in *plxna3* knockdown embryos ($N = 2$; $p < 0.0001$, $n = 17$; F) as compared to controls ($n = 13$). **G-H)** Representative images of *myh6*-expressing atria of control (G) and *plxna3* (H) morpholino-injected embryos. White arrow delineates spillover of ISH label into the ventricle. **I-J)** Representative images of *bmp4a* expression in control (I) and *plxna3* (J) morpholino-injected embryos. White arrow and arrowhead delineate spillover of ISH label into the ventricle and atria, respectively. **K)** The cardiomyocyte label *tnnt2a* shows a sharp reduction in the atrium of a control but not a *plxna3* MO embryo. **L-N)** Graphs showing average heart rate ($*p = 0.011$, L), cardiac output ($p = 0.095$, M), and ejection fraction ($p = 0.18$, N) ($N = 3$, $n = 9$ controls, $n = 10$ *plxna3* morphants). **O-Q)** Quantitation of cell size of DM-GRASP-labelled ventricular cardiomyocytes. A significant reduction is seen in cardiomyocyte area (O, $*p = 0.019$) and perimeter (P, $*p = 0.015$) in *plxna3*-deficient embryos as compared to controls, but not circularity (Q, $p = 0.15$). Measurements were made using $N = 3$, $n = 6$ control and $N = 3$, $n = 11$ *plxna3* morphant embryos, with 8-10 cells per embryo. **R)** Average area of the *bmp4a* expression domain in control ($n = 12$; $p = 0.028$) and *plxna3* morphants ($n = 7$). Error bars are SEM. Statistics represent the Mann-Whitney U test. Scale bar in A: 50 μm (A,B,D-D',K), 25 μm (C,G-J).

Supplemental Figure 1: *sema3f* expression and function in the developing heart. A)

Percentage of embryos with a severe heart edema at 48 hpf in WT and *sema3fb^{ca305}* embryos and those injected with 1 ng/ml or 3 ng/ml of an antisense morpholino oligonucleotide against the ATG start site of the *sema3fb* gene. Numbers in brackets represent number of embryos (N=2 independent experiments). **B)** Dorsal view of whole mount ISH for *sema3fb* at the 14 somite stage reveals that mRNA is expressed in the early heart field (yellow arrows) as marked by *hand2*. **C)** *sema3fa* shows little or no expression in the 48 hpf WT and *sema3fb* mutant heart.

Supplementary Figure 2: Early heart development occurs normally in the absence of Sema3fb. Dorsal (A-C) and lateral (D-F) views of 13 hpf WT (A,D), *sema3fb*^{ca305} (B, E), and *sema3fb*^{ca306} (C, F) embryos processed by whole mount ISH. **A-F)** *fgf8a* is expressed normally (N=2, n=10) in the heart field containing anterior lateral plate mesoderm as indicated by arrowheads in low magnification lateral (D-F) and high magnification dorsal (A-C) views. A, anterior; mhb, midbrain-hindbrain boundary; som, somites; tb, tailbud. Scale bar: 50 μ m for A-C, 100 μ m for D-F.

Supplemental Figure 3. Heart tube extension is delayed slightly in *sema3fb* mutant

embryos. Ventral views of WT, *sema3fb^{ca305}* and *sema3fb^{ca306}* 24 hpf embryos processed for whole mount ISH using cardiomyocyte specific riboprobes. **A-C)** *tnnt2a* ISH shows that the heart elongates in the WT to form a tube (N=3, n=15/17) (A). Progression into the extension stage is defective at 24 hpf in both *sema3fb* mutant alleles (N=3, 14/21 *sema3fb^{ca305}*; N=3, 15/21 *sema3fb^{ca306}*), with cone (B) and condensed (C) morphologies being exhibited. **D)**

Percent occurrence of the different cardiac morphologies. **E-G**) Ventricular cardiomyocyte specific *myh7* riboprobe also reveals elongation of WT hearts (N=3, n=17/20) (E), but that *sema3fb* mutant hearts exhibit cone or condensed morphologies (N=3, 12/19 *sema3fb*^{ca305}; N=3, 13/19 *sema3fb*^{ca306}) (F-G). **H**) Percent occurrence of the different cardiac morphologies. **I-K**) Atrial cardiomyocyte specific *myh6* riboprobe reveals that WT atria are tubularized (N=3, n=17/20) (I), but are primarily at the cone stage in both the *sema3fb* mutant alleles (N=3, 19/20 *sema3fb*^{ca305}; N=3, 11/18 *sema3fb*^{ca306}) (J-K). **L**) Percent occurrence of the different cardiac morphologies. **M-O**) Ventral views of embryos processed for whole mount ISH for the ventricle specific marker *myh7*. Heart tube elongation was scored for WT embryos at 23 hpf (N=1, n=9/10, M), and at 26 hpf for *sema3fb*^{ca305} (N=1, n=8/10, N) and *sema3fb*^{ca306} (N=1, n=9/10, O) embryos. **P-Q**) Ventral views of DM-GRASP whole mount immunostaining of embryos at 24 hpf show cranial ganglion (red asterisk) that has migrated and formed normally in WT (n=4, P) and *sema3fb*^{ca305} (n=4, Q) embryos, despite the heart of mutants showing a mild developmental delay (yellow arrowhead). **R**) Percentage occurrence of *myh7*-labeled heart morphologies between WT at 23 hpf, and *sema3fb*^{ca305} and *sema3fb*^{ca306} at 26 hpf. A: anterior; P: posterior. Scale bar: 100 μ m.

Supplemental Figure 4. Ventricle size is reduced significantly in *sema3fb* mutants. A-D)

Ventricle measurements were made from still images taken during video microscopy of WT and mutant hearts during diastole (A,C) and systole (B,D) at 72 hpf. Quantitation reveals that in mutants as compared to WT the ventricle diameter during diastole (A, **p=0.0017) and during systole (B, **p=0.0063) is significantly smaller, and ventricle length is significantly shorter at diastole (C, *p=0.048) and systole (D, *p=0.031). Error bars are SEM over 3 independent experiments (n=12 WT, n=20 *sema3fb*^{ca305}). Statistics represent the Mann-Whitney U test.

Supplemental Figure 5. Cardiomyocytes derived from the secondary heart field and cardiac neural crest are present in the developing *sema3fb* mutant heart. Whole mount ISH of 48 hpf hearts with riboprobes against the CNC marker *crip2* (A,B) and the secondary heart field marker *ltbp3a* (C,D). Similar labeling is seen in WT and *sema3fb^{ca305}* hearts for *crip2* (A-B, N=1, n=11 WT and N=1, n=11 *sema3fb^{ca305}*) and *ltbp3a* (C-D, N=1, n=6 WT and N=1, n=7 *sema3fb^{ca305}*). A: anterior; P: posterior. Scale bar: 50 μ m.

Supplemental Figure 6. Reduced cardiac chamber size is due to intrinsic loss of Sema3fb.

Heart explants dissected from embryos at 24 hpf and cultured for 24 hours before processing by whole mount ISH for the atrial cardiomyocyte marker *myh6*. The atria of *sema3fb*^{ca305} hearts (N=2, n=11/13) are smaller in size than WT atria (N=2, n=8/8). (C) Graph of atrial area shows a significant reduction in size in *sema3fb*^{ca305} (*p=0.025, Mann-Whitney U test) as compared to WT explants. Error bars represent SEM. Scale bar: 50 μm.

1 **Supplemental Figure 7: *nrp2b* is expressed by ventricular cardiomyocytes and is**
2 **required for normal heart development. A-C)** Dorsal views of whole mount ISH for
3 *sema3fb*, *nrp2b* and *plxna3* at 18 hpf, revealing expression in the developing heart field
4 (white arrowheads). **D-E)** *nrp2b* ISH signal in the presumptive ventricle as viewed
5 laterally in a 28 hpf embryo (D), and in a sagittal section (E') of a 36 hpf *Tg(flk:EGFP)*
6 embryo where endocardial cells that line the heart and neighbour the cardiomyocytes are
7 GFP positive (E). A blend of the two signals is shown in E''. **F-G)** *myh6* labelling of the
8 atrium in a control embryo (F) and one injected at the one-cell stage with an antisense
9 MO against *nrp2b* (G). **H-L)** ISH for various *plxna* receptors (I-L) show minimal signal
10 in the *tnnt2a* (H) labeled heart at 24 hpf.