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Supplemental Information

An Injury-Responsive Gata4 Program

Shapes the Zebrafish Cardiac Ventricle

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Supplemental Inventory

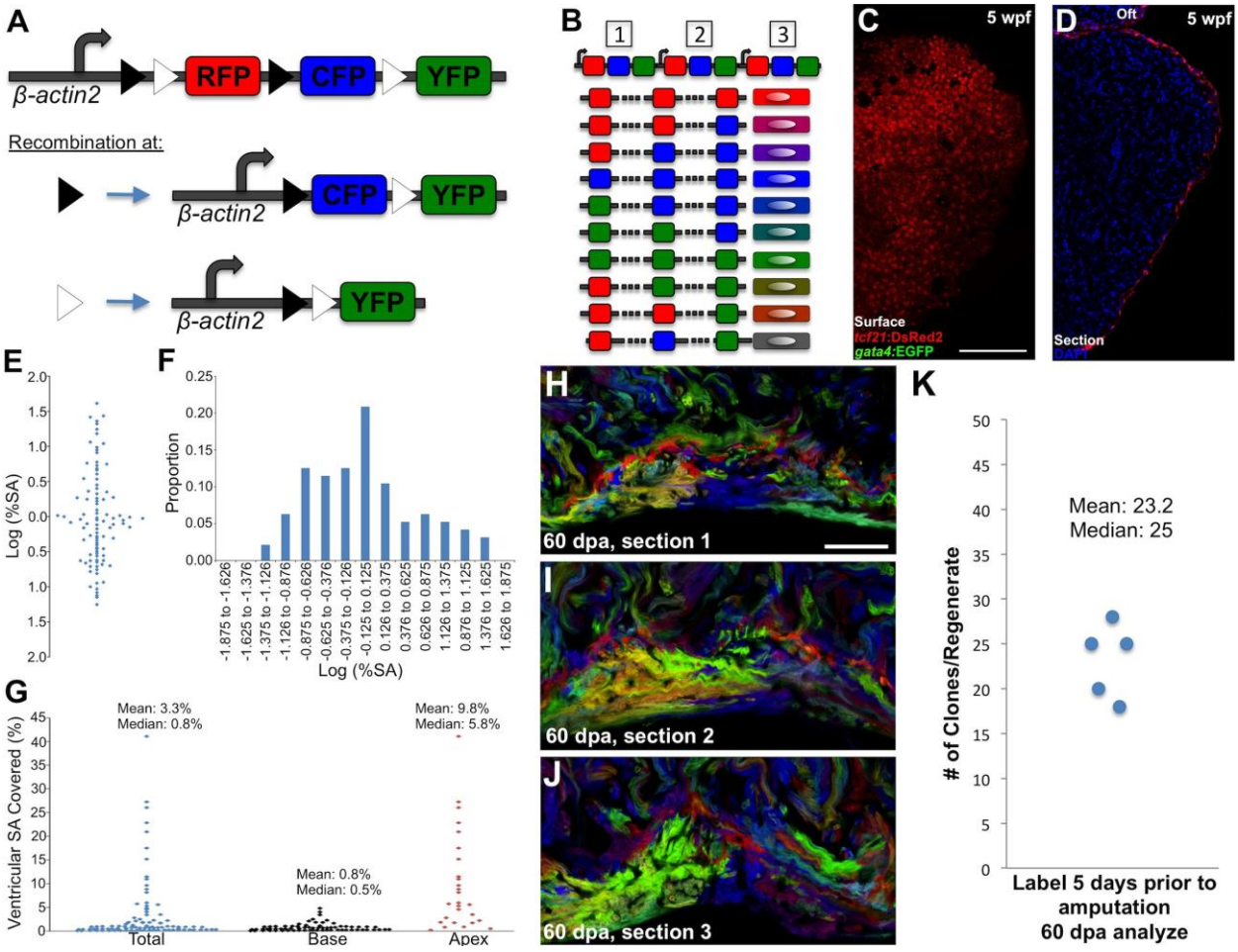

Figure S1. Brainbow Genetics, Early *gata4* Expression, Statistical Analysis of *gata4:ERCreER*; *priZm2* Clones, and Clonal Analysis of Regenerating Cardiomyocytes

(A) A cartoon of the *priZm* construct that drives expression of the Brainbow 1.0L cassette [1] with zebrafish *β-actin2* regulatory sequences. RFP is the initial reporter that is expressed in the absence of recombination. Recombination at the paired *lox2272* (black triangles) or *loxP* (white triangles) sites results in expression of CFP or YFP, respectively.

(B) Tandem insertions of a transgene at a single genetic locus are a common outcome of transgenesis. In the case of 3 transgenes, limited Cre-mediated stochastic recombination upon each Brainbow cassette will result in one of 10 possible outcomes that gives rise to 10 different colors. As the number of Brainbow cassettes increases, so do the possible number of unique outcomes and colors.

(C and D) Whole-mount (C) and section images (D) of a 5 wpf ventricle assessed for *tcf21:*DsRed2^{+} epicardial cells (red) and *gata4:*EGFP^{+} cardiomyocytes (green). *gata4*$^{+}$ cardiomyocytes are not detectable.

(E) A plot of the log-transformed % surface areas values from Figure 1G. The values have an average of -0.018 ± 0.665 (mean ± standard deviation). A Shapiro-Wilk test found that they differed significantly from a normal distribution, $p < 0.03$.

(F) Distribution of the log-normalized values from (E), indicating an upper tail that appears to represent a separate population of large clones.

(G) Percentage surface area (SA) occupied by cardiomyocyte clones generated after 6 wpf labeling in *gata4:ERCreER*; *priZm2* animals. All clone sizes were plotted (total), and then sizes were categorized regionally by location at the base or those that reached past the chamber midpoint (apex) (n = 69 basal and 27 apical clones from 9 ventricular halves).

(H-J) Adult *cmlc2:CreER; priZm* animals were labeled with 4-HT 5 days prior to amputation, and ventricular apices were resected 5 days later. Three serial 30-micron sections through a 60 dpa regenerate (H-J), in which multiple interwoven small clones can be visualized within the regenerate. Clonal representation changes markedly between sections. Dashed lines indicate the approximate plane of resection.

(K) Number of clones counted from 3 serial sections of the regenerates in 5 different *cmlc2:CreER; priZm* ventricles. The number of colors counted in these regenerates likely reaches the limit for this transgenic line. Scale bar = 100 µm (C and D); 50 µm (H-J)

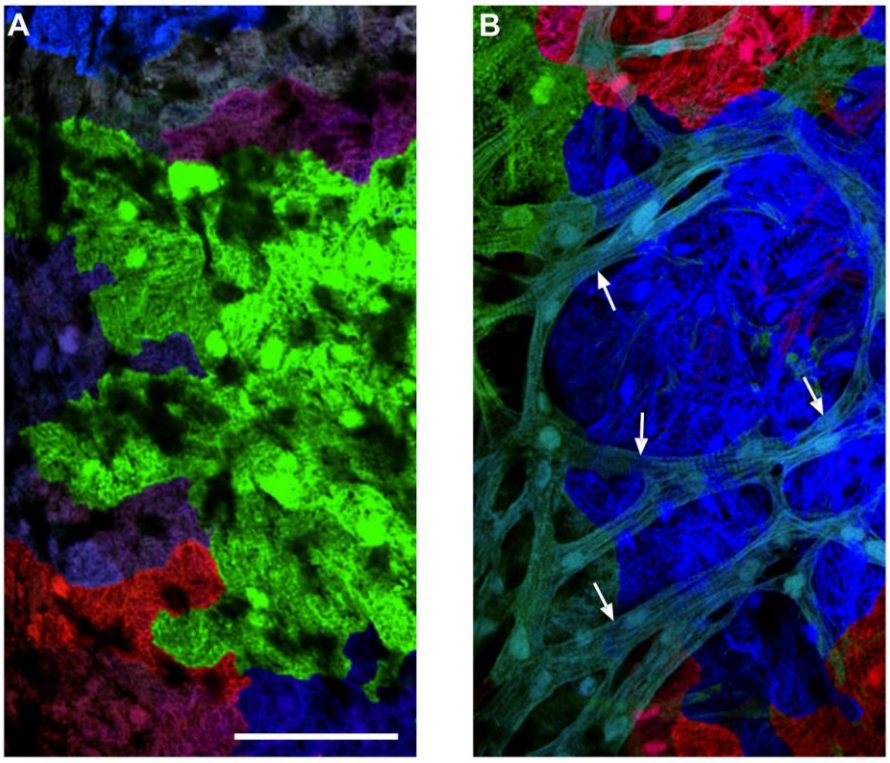

Figure S2. Morphological Differences between Cortical and Primordial Cardiomyocytes

(A) An image of a 6 wpf *cmlc2:CreER*; *priZm* ventricular surface, from an animal that had been labeled with 4-HT at 2 dpf. This imaged area is not covered with cortical muscle. The cardiomyocytes have limited sarcomeric structure.

(B) An image of a 10 wpf *cmlc2:CreER*; *priZm* ventricular surface, in which cyan colored cortical myocytes (arrows) are positioned on top of the primordial layer. Cortical myocytes are rod-shaped with clearer sarcomeric organization. Scale bars = 100 µm.

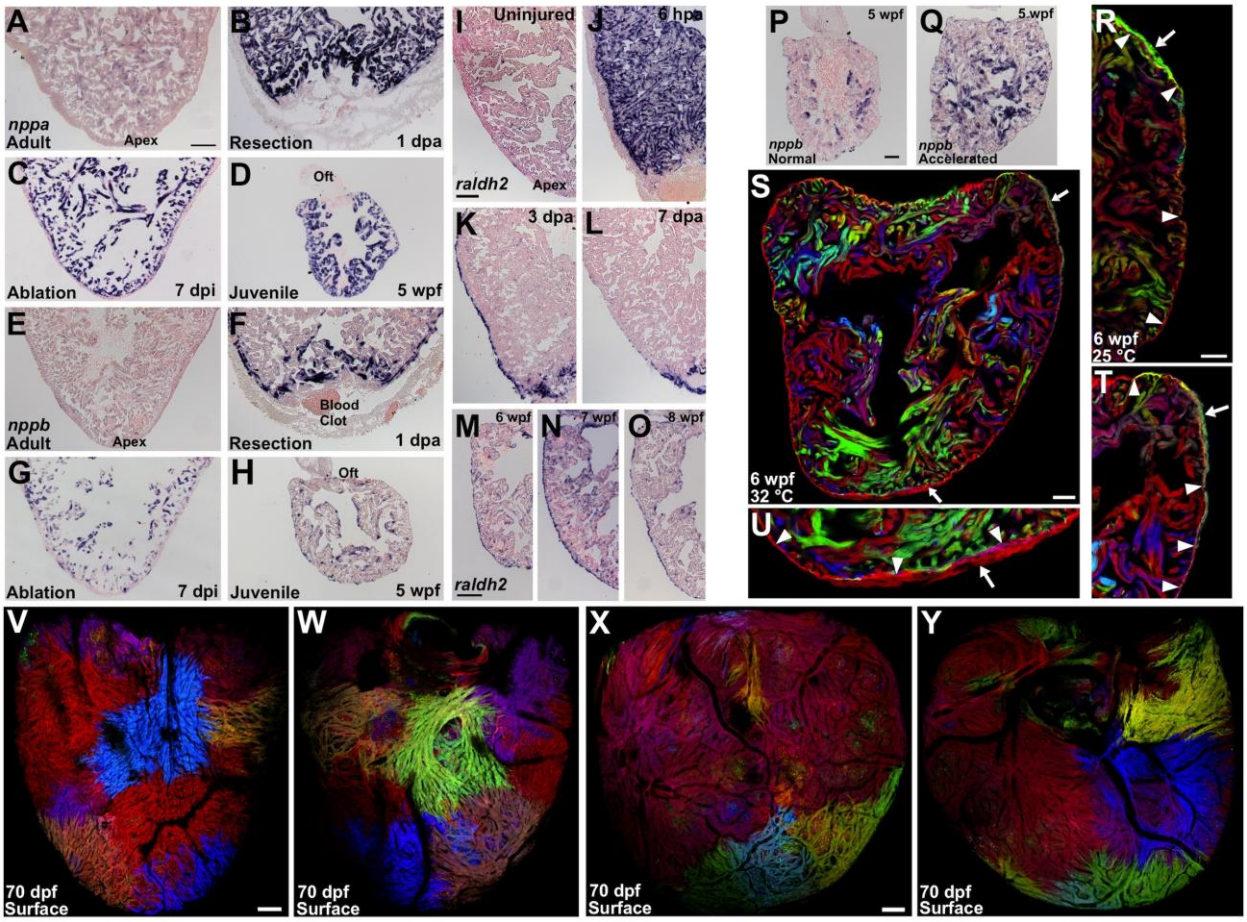

Figure S3. Injury and Stress Markers Are Expressed During Cortical Layer Formation, and Low-Density or High-Temperature Growth Experiments with *cmlc2:CreER; priZm* Animals

(A) Section of an adult ventricle stained by *in situ* hybridization for *nppa*, indicating low but detectable expression.

(B and C) By 1 day after apical resection (B) (dpa) or 7 days after diffuse genetic

muscle ablation (dpi) (C), *nppa* is strongly induced in cardiomyocytes.

(D) *nppa* expression is detected throughout the 5 weeks post-fertilization (wpf) ventricle (n = 8-14).

(E) Section of an adult ventricle stained by *in situ* hybridization for *nppb*, indicating no expression.

(F and G) By 1 day post amputation (dpa) (F) or 7 days after diffuse genetic muscle ablation (dpi) (G), *nppb* is induced in cardiomyocytes.

(H) *nppb* expression is detected throughout the 5 weeks post-fertilization (wpf) ventricle (n = 8-14).

(I-L) Sections of adult ventricles stained by *in situ* hybridization for *raldh2*. There is little or no detectable expression within an uninjured ventricle (I). After partial ventricular resection, *raldh2* is induced organ-wide in the endocardium by 6 hours post-amputation (hpa) (J) and organ-wide in the epicardium by 3 dpa (K). Expression in these cell layers localizes to the injury site by 7 dpa (L).

(M-O) During juvenile growth, *raldh2* is detected in both epicardial and endocardial tissues (n = 8-20). Oft, outflow tract.

(P and Q) Sections of 5 wpf ventricles stained by *in situ* hybridization for *nppb*, indicating higher expression and suggesting greater biomechanical stress in animals raised under low-density conditions that accelerate growth (n = 8-10).

(R) Juvenile animals grown at 25°C have an average of 1.1 ± 0.4 clones per heart at 6 wpf (n = 8). Here, a green cortical clone (arrow) can be seen at the base of heart external to the primordial muscle (arrowheads).

(S) Juvenile animals grown at 32°C have an average of 4.0 ± 0.6 clones per heart at 6 wpf (n = 8). Here, two cortical clones (arrows) can be seen at the base (gray) and the apex (red).

(T and U) Higher magnification of the two cortical clones from (S). The primordial layer is indicated by arrowheads.

(V and W) Animals were labeled at 2 dpf and analyzed at 70 dpf. Ventral (V) and dorsal (W) views of same ventricle in which many small cortical clones are visible.

(X and Y) A different ventricle. Many small cortical clones are visible, especially at the base of the ventricle. Scale bars = 100 μm (A-O and V-Y); 50 μm (P-U)

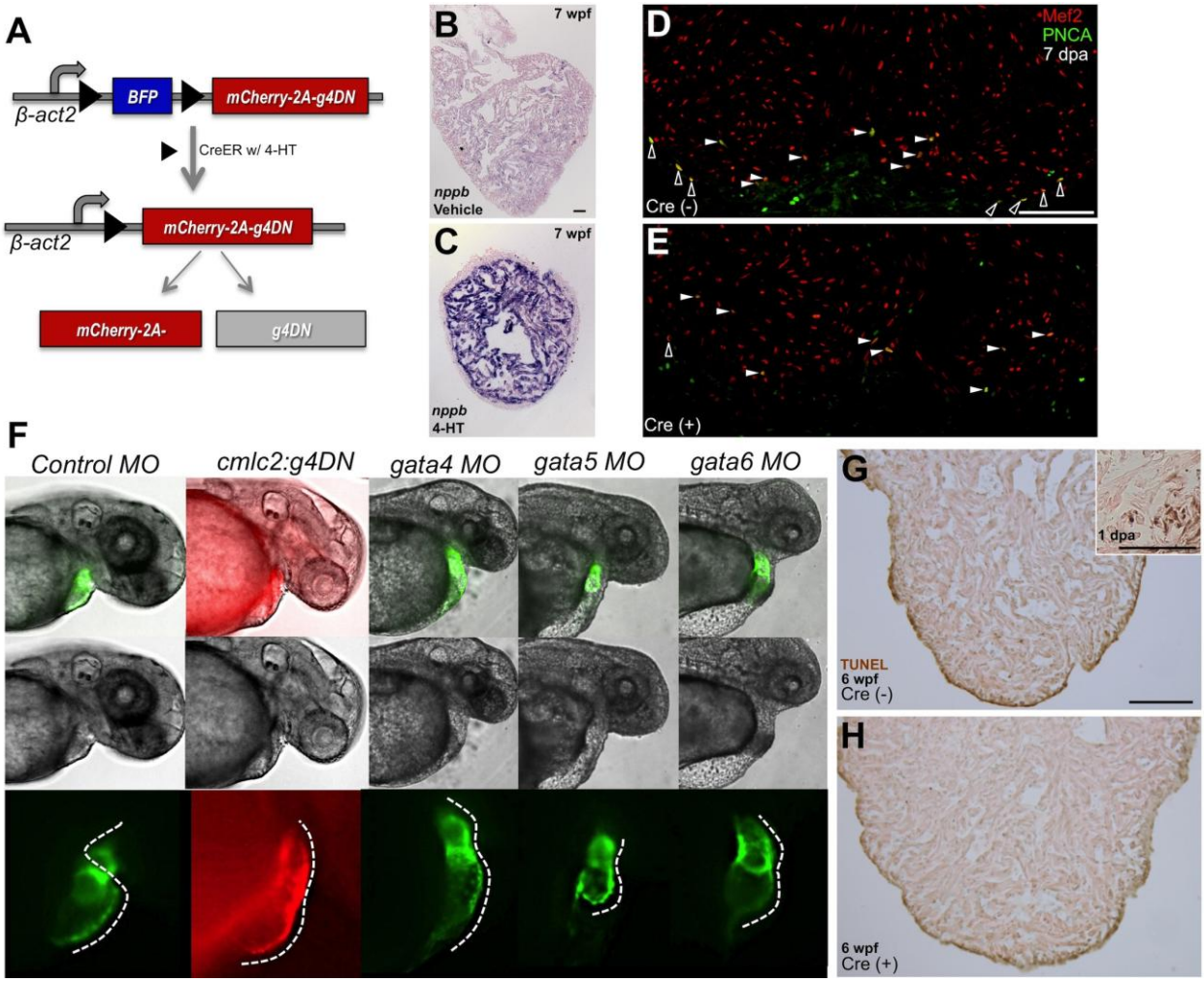

Figure S4. *g4DN* Overexpression Decreases Cortical Myocyte Proliferation and Phenocopies the *gata4* Morphant

(A) Cartoon describing the inducible expression of a dominant-negative version of Gata4. Cre-mediated recombination at *loxP* sites (black triangles) results in excision of Blue Fluorescent Protein (BFP) and expression of mCherry and g4DN, through a ribosomal 2A based translational skip.

(B and C) *β-act2:BSg4DN; cmlc2:CreER* animals were exposed to vehicle or 4-HT at 30 dpf and raised to 7 wpf. Ventricular sections were then stained by in situ hybridization for *nppb*; those expressing g4DN had higher cardiac *nppb* expression (n = 4).

(D and E) *β-act2:BSg4DN* animals with (D) or without (E) the *cmlc2:CreER* transgene were treated with 4-HT. Three days later, their ventricular apices were resected, and cardiomyocyte proliferation was assessed by Mef2/PCNA staining at 7 dpa. Examples of cortical (black) and trabecular (white) proliferating cardiomyocytes are indicated by arrowheads.

(F) Shown (left to right) is a representative control 48 hpf embryo, an embryo transgenic for the *cmlc2:g4DN* transgene (co-expressing a dominant-negative Gata4 isoform and TagRFP in cardiomycytes), or embryos that had been injected at the one cell stage with morpholinos targeting *gata4*, *gata5*, or *gata6*. Morphant embryonic hearts fluoresce green from a *cmlc2:EGFP* transgene. Panels show a fluorescent channel (top row), brightfield only (middle row), or a higher magnification view of the heart under fluorescence (bottom row). Dashed lines in the bottom row panels follow the outer curvature of the heart, indicating a normally looped heart tube in the control embryo (25 of 25 injected embryos), a non-looping distended linear heart tubes with chamber demarcation in the *cmlc2:g4DN* transgenic (28/28) and *gata4* morphant (34/36) embryos, a diminutive heart in the *gata5* morphant embryo (29/29), and a squat

hypoplastic linear heart in the *gata6* morphant embryo (27/31). *g4DN* transgenic embryos from independent founder lines each phenocopied the *gata4* morphant, while non-transgenic sibling embryos from these founders developed normal hearts (not shown).

(G and H) Apoptosis as assessed by TUNEL staining was not evident after induced cardiomyocyte *g4DN* expression from 5 to 6 wpf. Representative sections are shown, which indicated only rare TUNEL-positive cardiomyocytes in *β-act2:BSg4DN* animals with (H, n = 12) or without (G, n = 11) the *cmlc2:CreER* transgene. Some non-specific edge-staining was present in all samples. Inset shows the resection injury site of a 1 dpa adult ventricle (used as a positive control), indicating several TUNEL-positive cardiomyocytes.

Scale bars = 50 μm (B and C); 100 μm (D, E, G, and H).

Supplemental Experimental Procedures

Zebrafish

Wild-type or transgenic zebrafish of the hybrid EK/AB strain of the indicated ages were used for all experiments. All transgenic strains were analyzed as hemizygotes. Published transgenic strains or other alleles used in this study were *gata4:EGFP* (*Tg(gata4:EGFP)*^{ae1}) [2]; *cmlc2:CreER* (*Tg(cmlc2:CreER)*^{pd10}) [3] (used with *priZm* and *bactin2:loxp-mCherry-STOP-loxp-DTA*); *cmlc2:CreER* (*Tg(cmlc2:CreER)*^{pd13}) (used with *β-act2:BSg4DN*); *gata4:ERCreER* (*Tg(gata4:ERCreER)*^{pd39}) [3]; *tcf21:DsRed2* (*Tg(tcf21:DsRed2)*^{pd37}) [4]; *tcf21:nucEGFP* (*Tg(tcf21:nucEGFP)*^{pd41}) [5]; *priZm* (*Tg(β-act2:Brainbow1.0L)*^{pd49}) [6]; *priZm2* (*Tg(β-act2:Brainbow1.0L)*^{pd50}) (an independent *priZm* founder line); and *bactin2:loxp-mCherry-STOP-loxp-DTA* (*Tg(bactin2:loxP-mCherry-STOP-loxP-DTA176)*^{pd36}) [5]. Animal density was maintained from 28 dpf onward at 8-9 fish per 3 liters for growth and regeneration experiments, and at 5 fish per 10 liters for accelerated growth experiments. Ventricular resection surgeries were performed as described previously [7]. All experiments with zebrafish were performed in accordance with animal use guidelines at Duke University.

β-act2:BSg4DN Transgenic Animals

The *β*-act2:*RSG* plasmid [3] was digested with XhoI and the *DsRed* coding sequence was replaced by *TagBFP*. The resulting plasmid was further modified to replace *EGFP* by a linker sequence containing a PmeI site. A dominant-negative version of *gata4* (*sr-gata4*) [8] was PCR amplified and cloned in frame with mCherry2a to ensure bi-cistronic expression. The *mCherry-2a-sr-gata4* was cloned into the modified *β-act2*:*RSG* plasmid using the new PmeI site. This construct was co-injected into one-cell-stage wild-type embryos with I-SceI. Three founders were isolated and propagated. The full name of this transgenic line is *Tg(bactin2:loxP-mTagBFP-STOP-loxP-mCherry-2a-sr-gata4)*^{pd62}.

To generate zebrafish embryos with cardiomyocyte *g4DN* expression, pME-g4DN was recombined with p5E-cmlc2, p3E-IRES-TagRFP, and the Tol2 destination vector as described [9]. 30 pg of recombined vector and 30 pg of RNA encoding Tol2 recombinase were co-injected into single cell AB/Tu embryos that were raised to adults. Adult F0 founders were screened for transgene germline integration and 3 independent founders were identified. The F1 embryos derived from these mosaic founders were identified by the expression of TagRFP throughout the myocardium, while the non-transgenic RFP-negative siblings served as controls. Cardiac phenotypes of embryos from all 3 lines were indistinguishable and as presented in Figure S6. Morpholinos against *gata4*, *gata5*, or *gata6* were used as described [10, 11].

4-HT Treatment

cmlc2:CreER; priZm animals were labeled with 4-HT at 2 dpf as described previously [6]. To label adult *cmlc2:CreER; priZm* cardiomyocytes prior to regeneration assays, animals were treated with 1 μM 4-HT in aquarium water for 3 hours, from a 1 mM stock made in 100% ethanol. Three animals were placed in 80 ml of 4-HT solution for the treatment, and then returned to recirculating water.

To induce recombination in *cmlc2:CreER; β-act2:BSg4DN* fish at 30-35 dpf, 4 fish were placed in 40 ml of 5 μM 4-HT in aquarium water for 16 hours. For adult *cmlc2:CreER; β-act2:BSg4DN* animals, 3 fish placed in 80 ml of 5 μM 4-HT for 16 to 24 hours. Resections were performed 3 days after 4-HT treatment.

gata4:ERCreER; priZm2 juvenile fish were treated with 5 μM 4-HT for 6 hours, with 4 animals placed in 40 ml of 4-HT solution. Adult *gata4:ERCreER; priZm2* were labeled in 80 ml of 5 μM 4-HT for 16 hours.

For cardiomyocyte ablation in juvenile *cmlc2:CreER; bactin2:loxp-mCherry-STOP-loxp-DTA* fish, we treated animals with 0.5 or 1 μM 4-HT for 16 hours at 4 fish per 40 ml of aquarium water. Adult cardiomyocyte ablation injuries were performed as described [5].

Histological Methods

In situ hybridization (ISH) was performed using 10-12 μm cryosections and digoxygenin-labeled cRNA probes as described previously [7], with the aid of an InSituPro robot (Intavis). Zebrafish *nppa* cDNA was obtained from Open Biosciences. Zebrafish *nppb* cDNA was amplified from 72 hpf embryos using the primers 5'-TGCGGCCGCATGAAATCGCTTCA-3' and 5'-TGAATTCTCAGTTCTTCTTGGGACCT-3'. Acid Fuchsin-Orange G staining and immunofluorescence was performed on 10-12 μm cryosections as described [7]. TUNEL staining was performed as described [12]. Mef2/PCNA staining and quantification

was performed as described previously [13]. Primary antibodies used in this study were anti-Myosin heavy chain (F59, mouse; Developmental Studies Hybridoma Bank), anti-Mef2 (rabbit; Santa Cruz), anti-PCNA (mouse; Sigma). Secondary antibodies were Alexa Fluor 633 labeled goat anti-mouse IgG (H+L), Alexa Fluor 594 goat anti-rabbit IgG (H+L), and Alexa Fluor 488 goat anti-mouse IgG (H+L) from Invitrogen.

Imaging

Fluorescent images from all samples were acquired using a Leica SP5 AOBS or a Zeiss LSM 700 microscope as described previously [6]. For imaging of the ventricular surface, fixed ventricles were compressed between two coverslips to allow imaging of both sides. Confocal slices through whole-mounted ventricles were acquired by adjusting the Z-position until trabecular muscle could be visualized [6]. For some experiments, single confocal sections were acquired from 30 μm cryosections. Images from *priZm* samples were acquired using laser lines 458 nm, 515 nm, and 561 nm to excite CFP, YFP, and RFP, respectively. Channels were acquired sequentially, overlaid, and imported into Adobe Photoshop, where uniform adjustments were made to brightness and contrast. Clones were traced in ImageJ and quantified in μm^2. The percentage area occupied by a clone was calculated by dividing its measured area by the total surface area of both sides of the ventricle, or one side of the ventricle in the case of single-sided ventricle experiments. Fluorescence from BFP, GFP, and Alexa Fluor 633 were excited with a 405 nm, 488 nm, and 633 nm laser lines, respectively.

Supplemental References

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