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Mouse   1    MSLVGGFPHHPVVHHEGYPFAAAAAAAAAAAASRCSHEENPYFHGWLIGHPEMSPPDYSM  60
             MSLVGGFPHHPV+HH+GY FAAAAAA      SRC HEE PYFHGWLI HPEMSPPDY+M
Fish    1    MSLVGGFPHHPVMHHDGYSFAAAAAA------SRC-HEEPPYFHGWLISHPEMSPPDYTM  53

Mouse   61   ALSYSPEYASGAAGLDHSHYGGVPPGAGPPGLGGPRPVKRRGTANRKERRRTQSINSAFA  120
             A SYSPEY++GA GLDHSHYGGVP         GPR VKRR TANRKERRRTQSINSAFA
Fish    54   APSYSPEYSTGAPGLDHSHYGGVPGAGA--VGMGPRTVKRRPTANRKERRRTQSINSAFA  111

Mouse   121  ELRECIPNVPADTKLSKIKTLRLATSYIAYLMDLLAKDDQNGEAEAFKAEIKKTDVKEEK  180
             ELRECIPNVPADTKLSKIKTLRLATSYIAYLMD+L KD+QNG  EAFKAE KKTD KEE+
Fish    112  ELRECIPNVPADTKLSKIKTLRLATSYIAYLMDILDKDEQNGGTEAFKAEFKKTDAKEER  171

Mouse   181  RKKELNEILKSTVSSNDKKTKGRTGWPQHVWALELKQ  217
             RKKE+N++LKS+ SSNDKKTKGRTGWPQHVWALELKQ
Fish    172  RKKEMNDVLKSSGSSNDKKTKGRTGWPQHVWALELKQ  208
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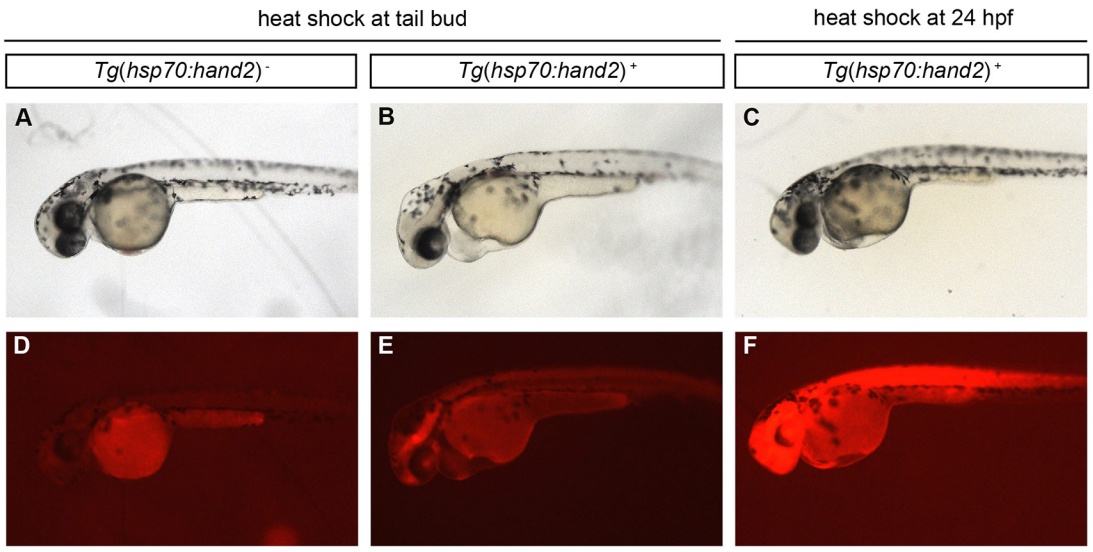
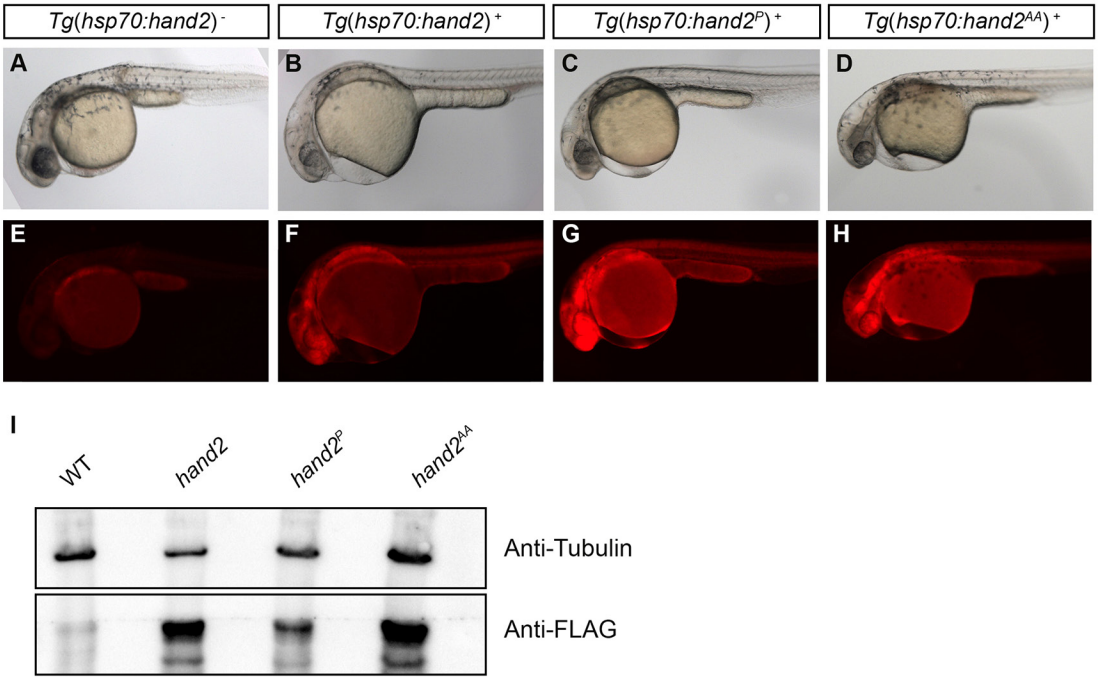
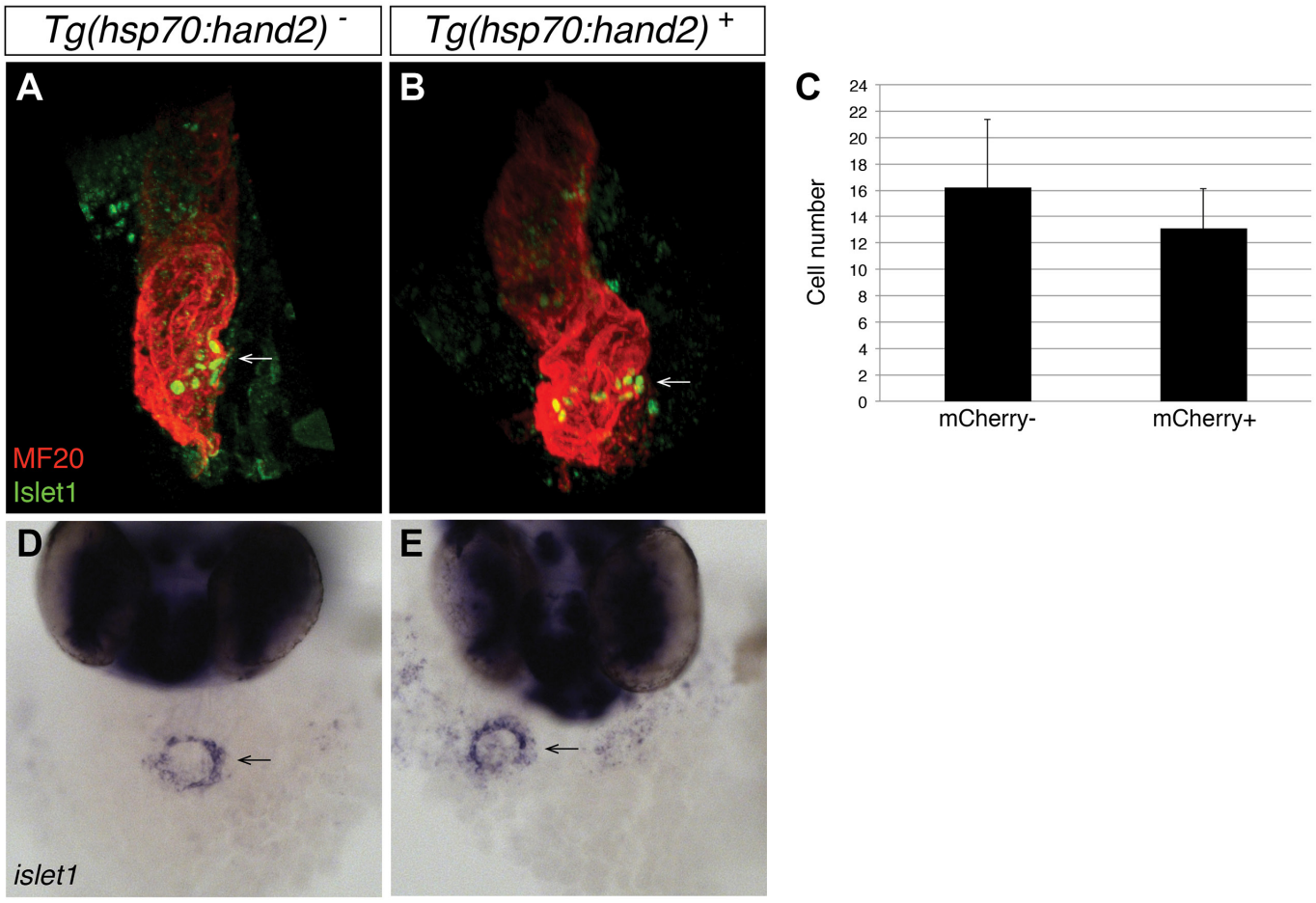
Supplemental Figure S1. Mouse and zebrafish Hand2 protein sequences are highly conserved. Alignment of amino acid sequences of mouse Hand2 (NP_034532.3; top row) and zebrafish Hand2 (AAI65015.1; bottom row), generated through pairwise BLASTP (Altschul et al., 1997). Identical and similar amino acids are indicated in the middle row. The three arginines replaced in the DNA binding-deficient form of Hand2 (Hand2 EDE) are shown in red. The phenylalanine replaced in the dimerization-deficient form of Hand2 (Hand2 P) is shown in green. The threonine and serine replaced in the phosphorylation-deficient form of Hand2 (Hand2AA) are shown in blue.



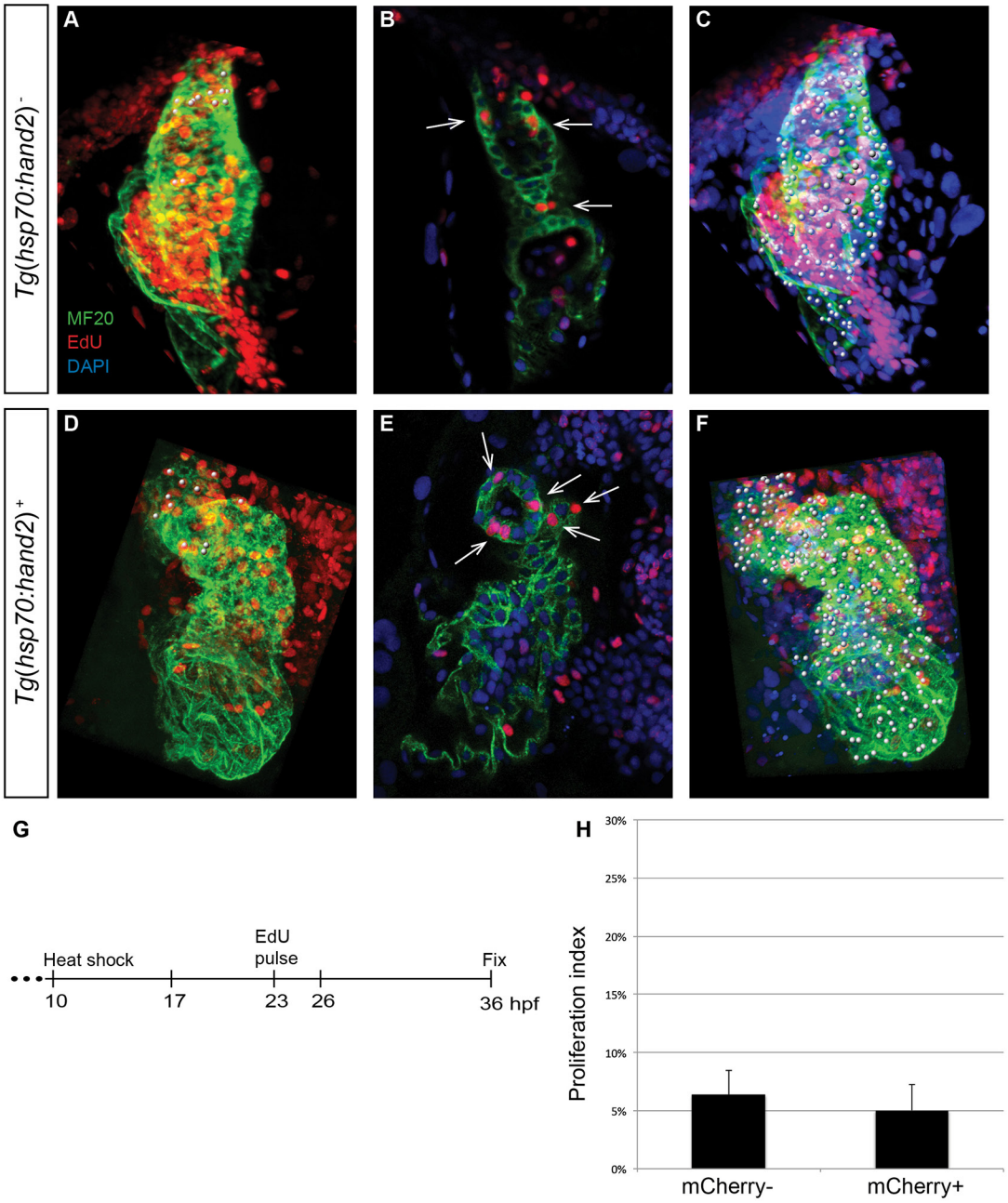
Supplemental Figure S2. Induction of *hand2* overexpression at 10 hpf, but not at 24 hpf, produces a morphologically evident cardiac phenotype. (A-F) Lateral views of live embryos at 36 hpf depict (A-C) embryonic morphology and (D-F) mCherry fluorescence in (A,D) a nontransgenic embryo and (B,C,E,F) embryos carrying the transgene *Tg(hsp70:FLAG-hand2-2A-mCherry)* (abbreviated as *Tg(hsp70:hand2)*), after heat shock at (A,B,D,E) 10 hpf (tail bud) or (C,F) 24 hpf. Heat shock of transgenic embryos at 10 hpf leads to pericardial edema (B), and residual mCherry fluorescence is still evident in these embryos at 36 hpf (E). In contrast, edema is not observed in transgenic embryos after heat shock at 24 hpf (C); mCherry fluorescence remains pervasive in these embryos at 36 hpf (F).



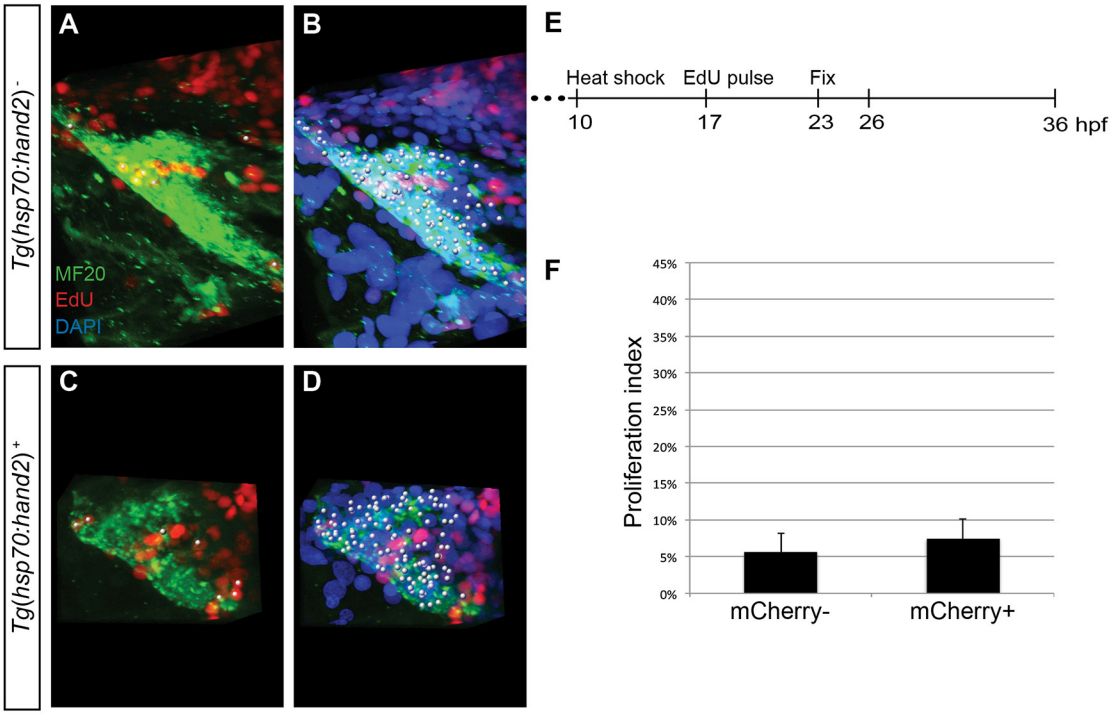
Supplemental Figure S3. Levels of protein production in different transgenic lines. (A-H) Lateral views of live embryos at 30 hpf depict bright field images of representative embryos from different transgenic lines (A-D), along with corresponding mCherry fluorescence (E-H). (E) No mCherry fluorescence is induced in heat-shocked nontransgenic embryos. (F-H) mCherry fluorescence is readily detectable in representative embryos carrying the transgenes (F) *Tg(hsp70:FLAG-hand2-2A-mCherry)* (*Tg(hsp70:hand2)*), (G) *Tg(hsp70:FLAG-hand2^{P}-2A-mCherry)* (*Tg(hsp70:hand2^{P})*), and (H) *Tg(hsp70:FLAG-hand2^{AA}-2A-mCherry)* (*Tg(hsp70:hand2^{AA})*). (**I**) Western blot analysis compares levels of FLAG-tagged Hand2 protein in different transgenic lines. Embryos were deyolked and lysates were prepared as previously described (Link et al., 2006), and blots were probed with either a monoclonal anti-FLAG M2 antibody (F1804, Sigma, 1:2000) or a monoclonal anti-α-Tubulin antibody (T6728, Sigma, 1:10,000), followed by a rabbit anti-mouse IgG HRP-conjugated secondary antibody (ab97046**,** Abcam, 1:10,000). Proteins were visualized using SuperSignal West Femto Chemiluminescent Substrate (Thermo Scientific). Each lane contains lysate from 15 embryos at 36 hpf (2 hours following heat shock), and the lanes compare protein levels in nontransgenic embryos (WT), *Tg(hsp70:hand2)* embryos *(hand2)*, *Tg(hsp70:hand2^{P})* embryos *(hand2^{P})*, and *Tg(hsp70:hand2^{AA})* embryos *(hand2^{AA})*. All three transgenic lines contain a ~23 kD FLAG-Hand2 protein, with comparable levels in *Tg(hsp70:hand2)* and *Tg(hsp70:hand2^{AA})* embryos and lower levels in *Tg(hsp70:hand2^{P})* embryos.



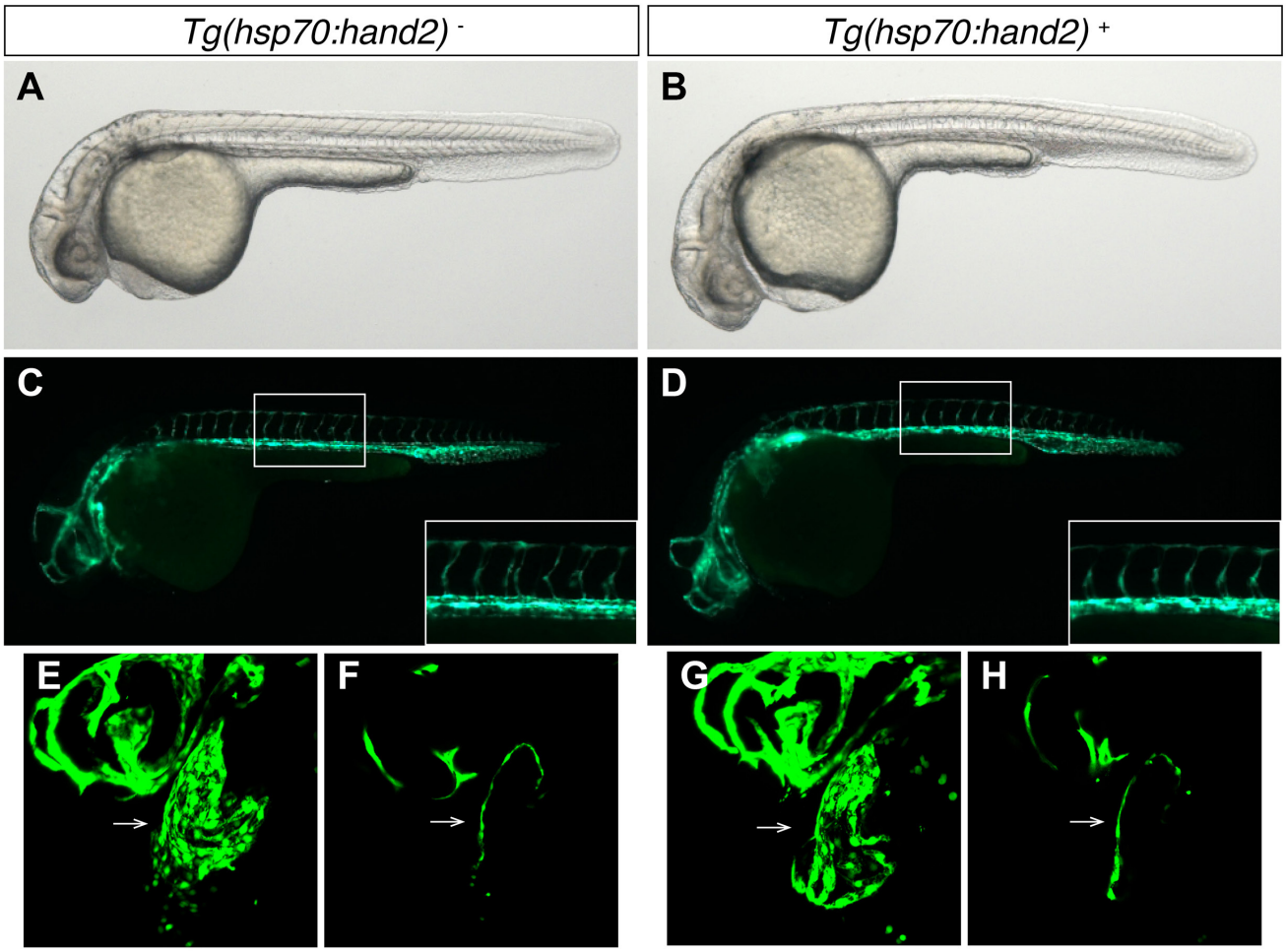
Supplemental Figure S4. Normal *islet1* expression at the venous pole in embryos overexpressing *hand2*. (A-B) Immunofluorescence at 36 hpf for MF20 (red, visible throughout the heart) and Islet1 (green, visible in the nuclei of a subset of atrial cells). Frontal views, dorsal to the top; arrows point to the Islet1-positive population at the venous pole of the atrium. Following heat shock at 10 hpf, the population of Islet1-positive cells at the venous pole appears similar in hearts from (A) nontransgenic embryos and (B) *Tg(hsp70:hand2)* embryos. **(C)** Bar graph compares average number of Islet1-positive cardiomyocytes at 36 hpf in nontransgenic embryos and *Tg(hsp70:hand2)*embryos, following heat shock at 10 hpf. Error bars indicate standard deviation; no significant difference is observed between these two data sets (n=12, p=0.09). (**D-E**) In situ hybridization depicts *islet1* expression at 36 hpf, following heat shock at 10 hpf. Frontal views, dorsal to the top; arrows point to the ring of *islet1*-expressing cells at the venous pole of the atrium. Expression patterns are similar in (D) nontransgenic embryos and (E) *Tg(hsp70:hand2)* embryos.



Supplemental Figure S5. No evident influence of *hand2* overexpression on cardiomyocyte proliferation after initial heart tube assembly. (A-F) EdU incorporation in hearts of (A-C) nontransgenic and (D-F) *Tg(hsp70:hand2)* embryos at 36 hpf, following heat shock at 10 hpf and EdU pulse at 23 hpf; (A,C,D,F) partial reconstructions of confocal z-stacks with ventricle up and (B,E) representative single slices. Dots, arrows, and color schemes are as described for Fig. 5A-F. Note that the nontransgenic heart shown (A) contains a number of EdU-positive blood cells that were trapped during fixation; EdU-positive blood cells are less commonly observed within the hearts of *hand2*-overexpressing embryos (D), due to their impaired circulation. **(G)** Timeline of experimental design. **(H)** Bar graph compares proliferation indexes in nontransgenic (mCherry-negative) and *Tg(hsp70:hand2)* (mCherry-positive) embryos, as in Fig. 5H. No change in proliferation index is seen in *hand2*-overexpressing embryos (n=8-11; p=0.196).



Supplemental Figure S6. No evident influence of *hand2* overexpression on cardiomyocyte proliferation within the heart tube at 23 hpf. (A-D) EdU incorporation in hearts of (A,B) nontransgenic and (C,D) *Tg(hsp70:hand2)* embryos at 23 hpf, following heat shock at 10 hpf and EdU pulse at 17 hpf; partial reconstructions of confocal z-stacks. Images depict the elongating cardiac cone, positioned with its arterial end toward the top. (A,C) White dots indicate EdU-positive (red) cells that are also MF20-positive (green) differentiated cardiomyocytes. (B,D) White dots indicate all nuclei (DAPI, blue) of myocardial cells, including both EdU-positive and EdU-negative cardiomyocytes. **(E)** Timeline of experimental design. **(F)** Bar graph compares proliferation indexes in nontransgenic (mCherry-negative) and *Tg(hsp70:hand2)* (mCherry-positive) embryos, as in Fig. 5H. No change in proliferation index is seen in *hand2*-overexpressing cardiomyocytes (n=10-11; p=0.252). Similarly, when we assessed EdU incorporation in *hand2*-overexpressing embryos at 26 hpf, following heat shock at 10 hpf and EdU pulse at 14 hpf, we did not see an increased proliferation index in *hand2*-overexpressing cardiomyocytes (proliferation index of 28 ± 4% in nontransgenic embryos compared to proliferation index of 27 ± 4% in *hand2*-overexpressing embryos; n=7-10, p=0.58).



Supplemental Figure S7. Endocardium is present in embryos overexpressing *hand2*. (A-H) Lateral views of live embryos at 30 hpf depict (A-B) embryonic morphology and (C-H) expression of the transgene *Tg(kdrl:GRCFP)* in the vasculature of (A,C,E,F) nontransgenic embryos and (B,D,G,H) *Tg(hsp70:hand2)* embryos, following heat shock at 10 hpf. (C-D) General vascular patterning and sprouting of intersomitic vessels (inset) is intact in embryos overexpressing *hand2* (D). (E-H) Partial reconstructions of confocal z-stacks (E,G) and representative single slices (F,H) highlight the endocardium (arrows): just as in nontransgenic embryos (E,F), a thin layer of endocardial tissue lines the entire heart tube in embryos overexpressing *hand2* (G,H).

SUPPLEMENTAL REFERENCES

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* 25, 3389-3402.

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