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Figures and figure supplements

Nrg1 is an injury-induced cardiomyocyte mitogen for the endogenous heart regeneration program in zebrafish

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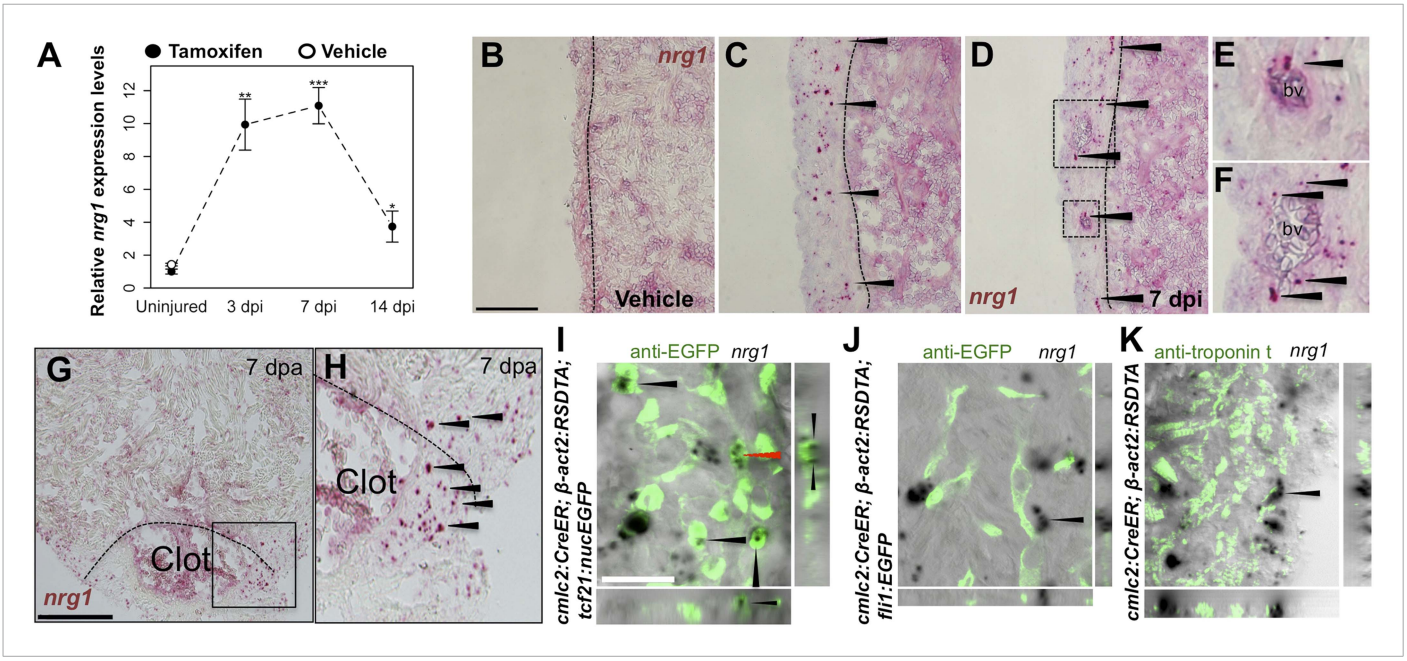

Figure 1. Induction of Nrg1 after cardiac injury. (**A**) Time course of *nrg1* induction in cardiac ventricles following severe genetic ablation of cardiomyocytes. *nrg1* mRNA levels were assessed by qPCR at 3, 7, and 14 days after ablation injury in tamoxifen-treated *cmlc2:CreER; β-act2:RSDTA* animals relative to control *cmlc2:CreER* animals (closed circles). *cmlc2:CreER; β-act2:RSDTA* (open circle) vehicle-treated animals serve as an additional control. Data are presented as mean ± SE. *p < 0.05, **p < 0.01, ***p < 0.001, Student's *t*-test, two-tailed. (**B**–**F**) Section images of in situ hybridization experiments assessed for *nrg1* expression in uninjured ventricles (**B**) or at 7 days after induced cardiomyocyte ablation (**C** and **D**). Dashed lines delineate the ventricular wall from the trabecular compartment. Higher magnification of boxes in (**E**) and (**F**) reveal *nrg1* signals surrounding vessels (bv). Arrowheads indicate examples of RNAscope signals. Scale bar represents 100 μm (**A** and **C**). (**G** and **H**) Section images of RNAScope in situ hybridization analysis for *nrg1* expression at 7 days after ventricular resection surgery. Image in (**H**) is a higher magnification of box in (**G**). Arrowheads indicate examples of RNAscope signals. Scale bar represents 100 μm. (**I**–**K**) Confocal slice images, with accompanying orthogonal views, of *nrg1* expression colocalized with *tcf21*:nucEGFP (**I**), *fli1*:EGFP (**J**), or cardiac muscle (Troponin, **K**). Arrows point to RNAscope signal, and red arrows indicate area for orthogonal views. Scale bar represents 20 μm.

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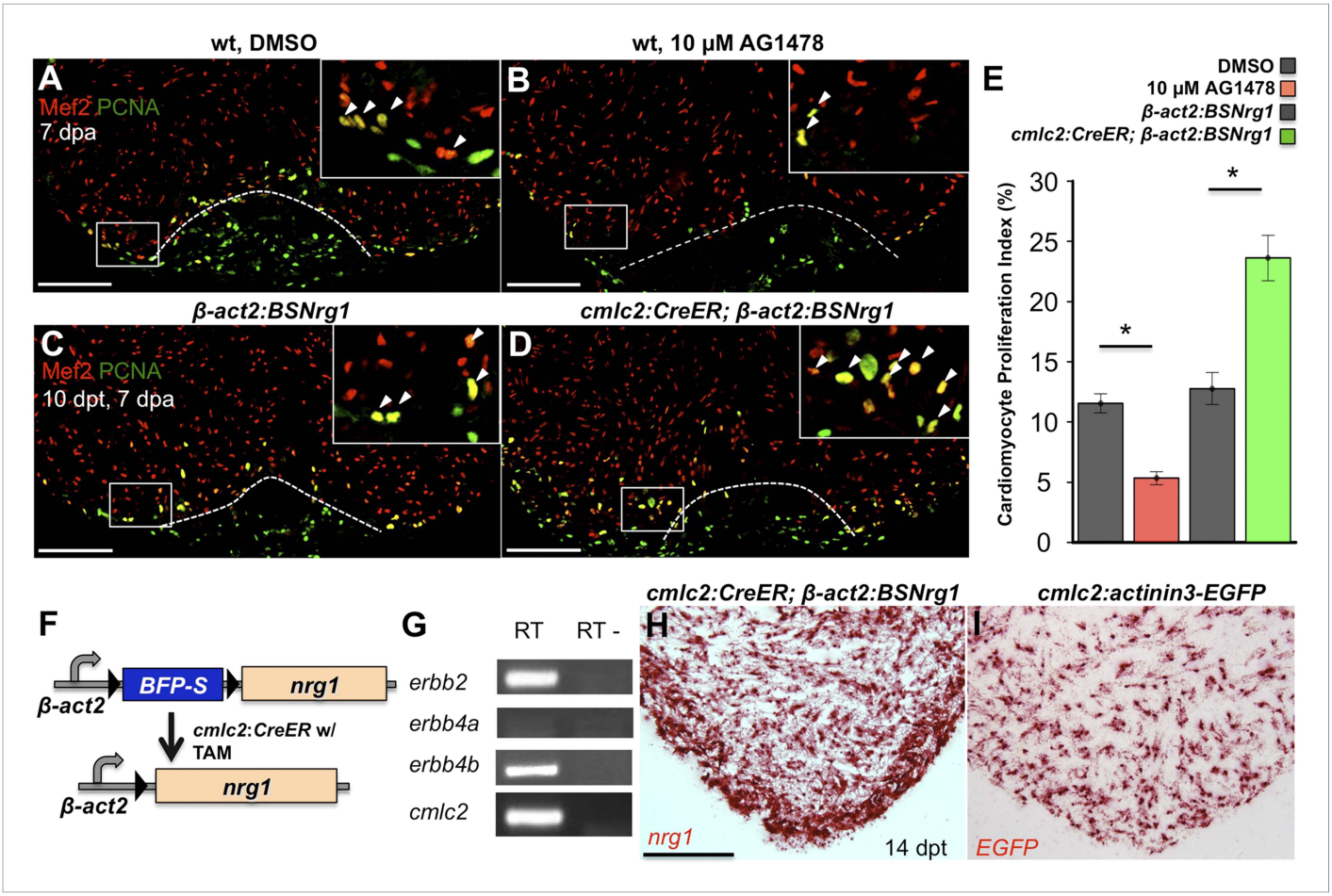

Figure 2. Nrg1 signaling modulates cardiomyocyte proliferation during regeneration. (**A** and **B**) Section images of injured ventricular apices of animals treated from 6 to 7 dpa with DMSO (**A**) or 10 μM AG1478 (**B**) and stained for Mef2^{+}PCNA^{+} cells (arrowheads). Wounds are indicated by dotted lines. Scale bar represents 100 μm. (**C** and **D**) Section images of 7 dpa ventricular apices of control *β-act2:BSNrg1* (**C**) or *cmlc2:CreER; β-act2:BSNrg1* (**D**) animals treated with tamoxifen at 3 days before injury, stained for Mef2^{+}PCNA^{+} cells (arrowheads). Scale bar represents 100 μm. (**E**) Quantification of cardiomyocyte proliferation at 7 dpa. DMSO-treated wild-type clutchmates (n = 22) were used as controls for 10 μM AG1478 treatment (n = 20), and tamoxifen-treated *β-act2:BSNrg1* clutchmates (n = 15) were controls for *cmlc2:CreER; β-act2:BSNrg1* (n = 18) animals. Data are represented as mean ± SEM. *p < 0.05, Mann–Whitney Ranked Sum Test. (**F**) Cartoon schematic of *β-act2:BSNrg1* transgene. (**G**) RT-PCR results for *erbb2*, *erbb4a*, and *erbb4b*, indicating the presence of *erbb2* and *erbb4b* messages in the uninjured adult ventricle. *cmlc2* is shown as a control. (**H**) Section image of RNAscope in situ hybridization analysis for *nrg1* expression at 14 days after tamoxifen-released expression in uninjured *cmlc2:CreER; β-act2:BSNrg1* ventricles. (**I**) Section image of RNAscope in situ hybridization analysis for *EGFP* expression in uninjured *cmlc2:actinin3-EGFP* ventricles, used as a control to detect transgenic signals. Scale bar represents 100 μm.

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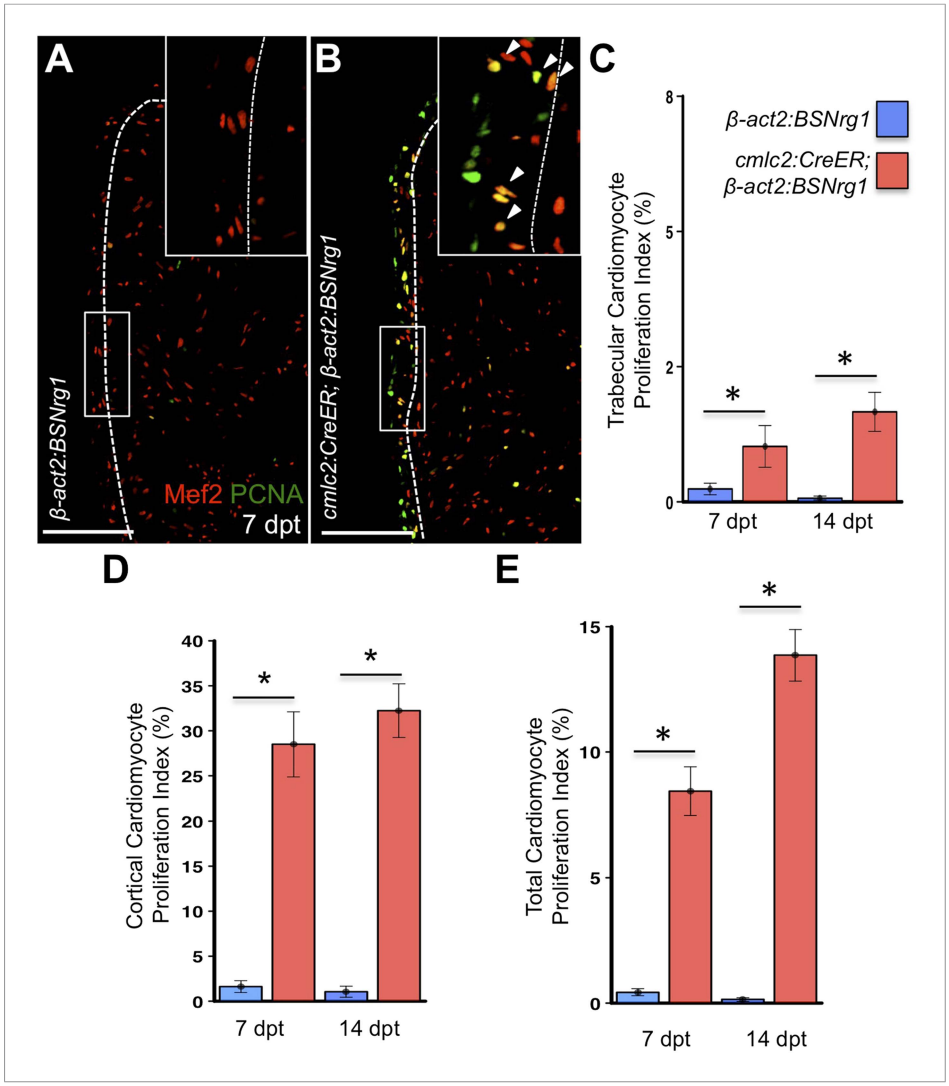

Figure 3. Nrg1 reactivation without injury induces proliferation of ventricular wall cardiomyocytes. (**A** and **B**) Section images from uninjured *cmlc2:CreER; β-act2:BSNrg1* and control ventricles at 7 days post-tamoxifen treatment (dpt), stained for Mef2^{+}PCNA^{+} cells. Insets show high-zoom views of the boxed regions, and arrowheads indicate Mef2^{+} PCNA^{+} nuclei. Dashed lines delineate cortical (wall) from trabecular muscle. Scale bars represent 100 μm. (**C**) Quantification of cardiomyocyte proliferation in *cmlc2:CreER; β-act2:BSNrg1* and controls in the trabecular muscle compartment at 7 (n = 8, 9) and 14 dpt (n = 10, 10). Data are represented as mean ± SEM. *p < 0.05, Mann–Whitney Ranked Sum Test. (**D**) Quantification of cardiomyocyte proliferation in cortical muscle at 7 (n = 8, 9) and 14 dpt (n = 10, 10), from groups in (**A** and **B**). Data are represented as mean ± SEM. *p < 0.05, Mann–Whitney Ranked Sum Test. (**E**) Quantification of total cardiomyocyte proliferation at 7 (n = 8, 9) and 14 dpt (n = 10, 10), from groups in (**C** and **D**). Data are represented as mean ± SEM. *p < 0.05, Mann–Whitney Ranked Sum Test.

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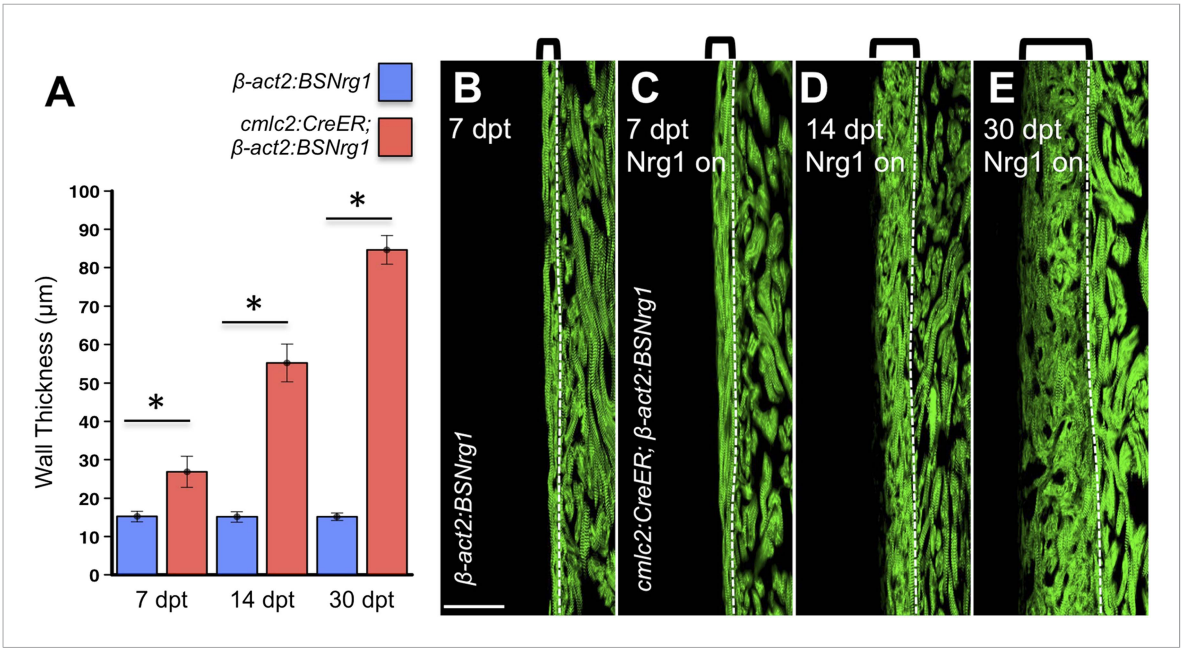

Figure 4. Nrg1-induced cardiomyocyte proliferation expands the ventricular wall. (**A**) Quantification of cortical muscle thickness at 7 (n = 8, 9), 14 (n = 10, 11), and 30 dpt (n = 11, 11). Data are represented as mean ± SEM. *p < 0.05, **p < 0.01, Student's *t*-test, two-tailed. (**B**–**E**) Section images of *cmlc2:CreER; β-act2:BSNrg1* (Nrg1 on) and control ventricles from 7 to 30 dpt, using animals also transgenic for *cmlc2:actinin3-EGFP* to indicate sarcomere organization. Brackets indicate cortical muscle, and dashed lines delineate cortical from trabecular muscle. Scale bar represents 100 μm.

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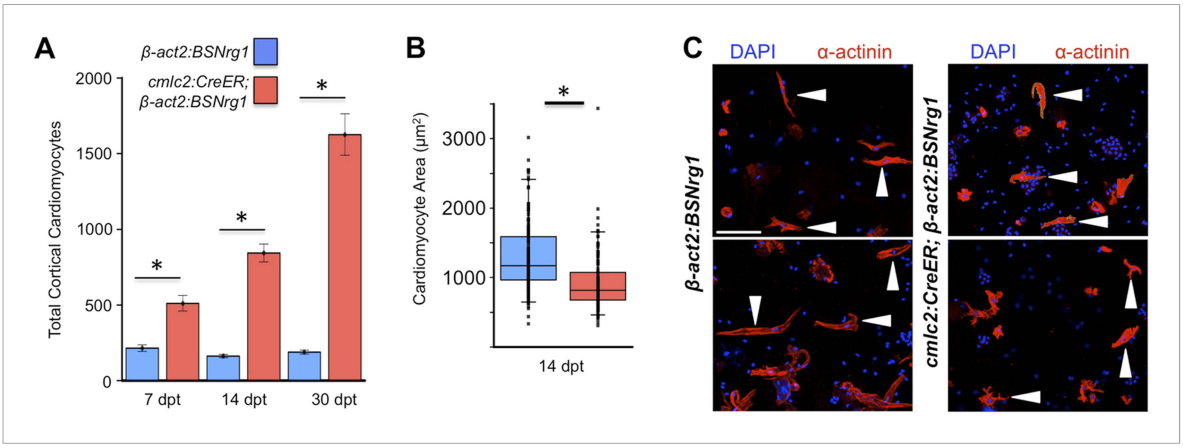

Figure 5. Nrg1 induces a hyperplastic, not hypertrophic, response. (**A**) Quantification of total ventricular wall cardiomyocytes in *cmlc2:CreER; β-act2:BSNrg1* animals and controls at 7 (n = 8, 9) and 14 dpt (n = 10, 10). Data are represented as mean ± SEM. *p < 0.05, Student's *t*-test, two-tailed. (**B**) Quantification of cardiomyocyte area in *cmlc2:CreER; β-act2:BSNrg1* animals and controls at 14 dpt. Data are represented as mean ± SD, with all data points represented. *p < 0.05, Student's *t*-test, two-tailed. (**C** and **D**) Confocal images of dissociated cardiomyocytes from *cmlc2:CreER; β-act2:BSNrg1* and controls at 14 dpt (n = 124, 172). Only cardiomyocytes with visible sarcomeres and nuclei were measured. Examples of quantified cells are marked with arrows. Scale bar represents 100 μm.

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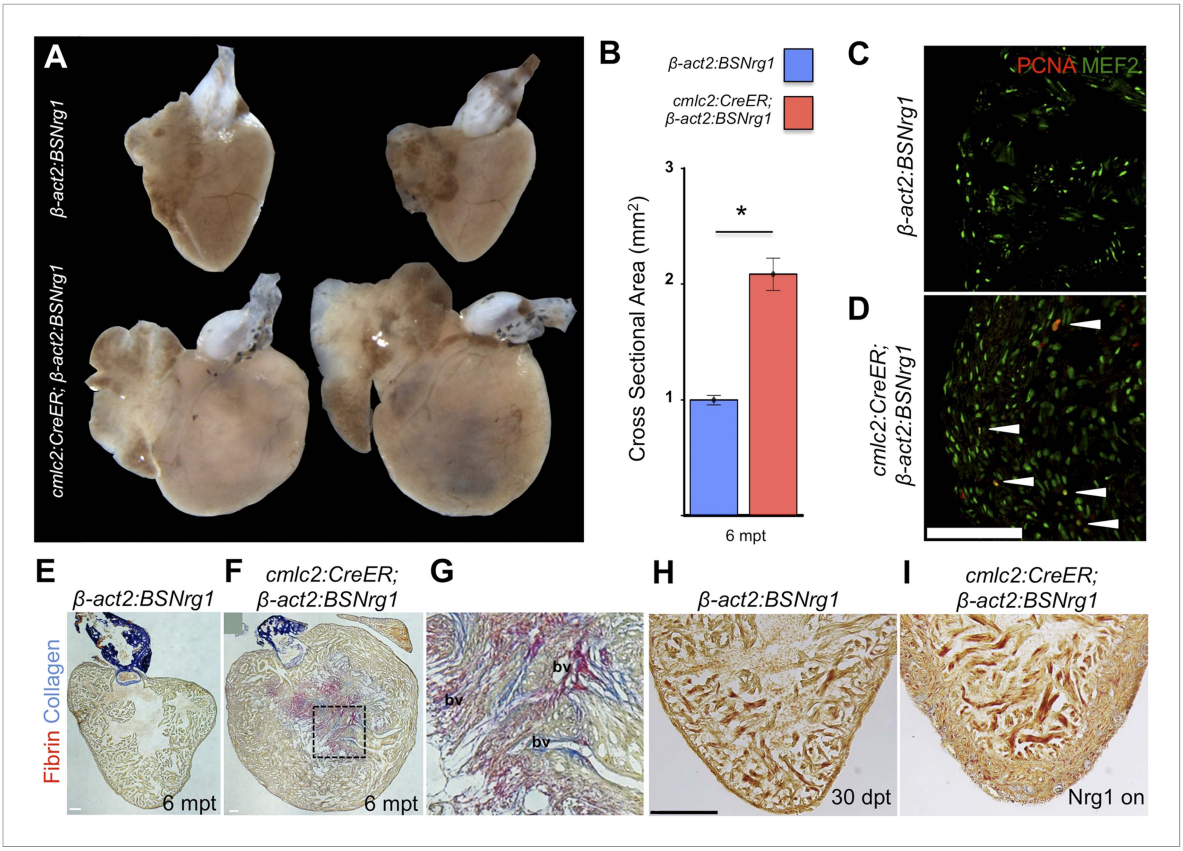

Figure 6. Nrg1-induced hyperplasia causes cardiomegaly. (**A**) Whole-mount images of *cmlc2:CreER; β-act2:BSNrg1* and control ventricles at 6 months post-tamoxifen treatment. (**B**) Quantification of the cross-sectional surface area of *cmlc2:CreER; β-act2:BSNrg1* (n = 9) and control ventricles (n = 10) 6 months post-treatment, revealing cardiomegaly effects of *nrg1* overexpression. Data are represented as mean ± SEM. *p < 0.05, Student's *t*-test, two-tailed. (**C** and **D**) Section images of ventricular walls of 6 mpt control *β-act2:BSNrg1* (**C**) or *cmlc2:CreER; β-act2:BSNrg1* animals (**D**) stained for Mef2^{+}PCNA^{+} cells (arrowheads). Scale bar represents 100 μm. (**E**) Section images of control *β-act2:BSNrg1* ventricles stained with Acid-Fuchsin Orange G (AFOG), revealing minimal collagen (blue), or fibrin deposition (red). Scale bar represents 100 μm. (**F** and **G**) Section images of *cmlc2:CreER; β-act2:BSNrg1* ventricles stained with AFOG, revealing collagen (blue) and fibrin deposition (red) in the inner portions of the thickened ventricular wall. Image in (**G**) is a high-zoom view of box in (**F**) and also indicates two examples of large coronary vessels (bv). (**H** and **I**) Acid-Fuchsin Orange (AFOG) staining reveals minimal fibrosis in *cmlc2:CreER; β-act2:BSNrg1* ventricle at 30 dpt despite the thickened ventricular wall (n = 7, 7). Scale bar represents 100 μm.
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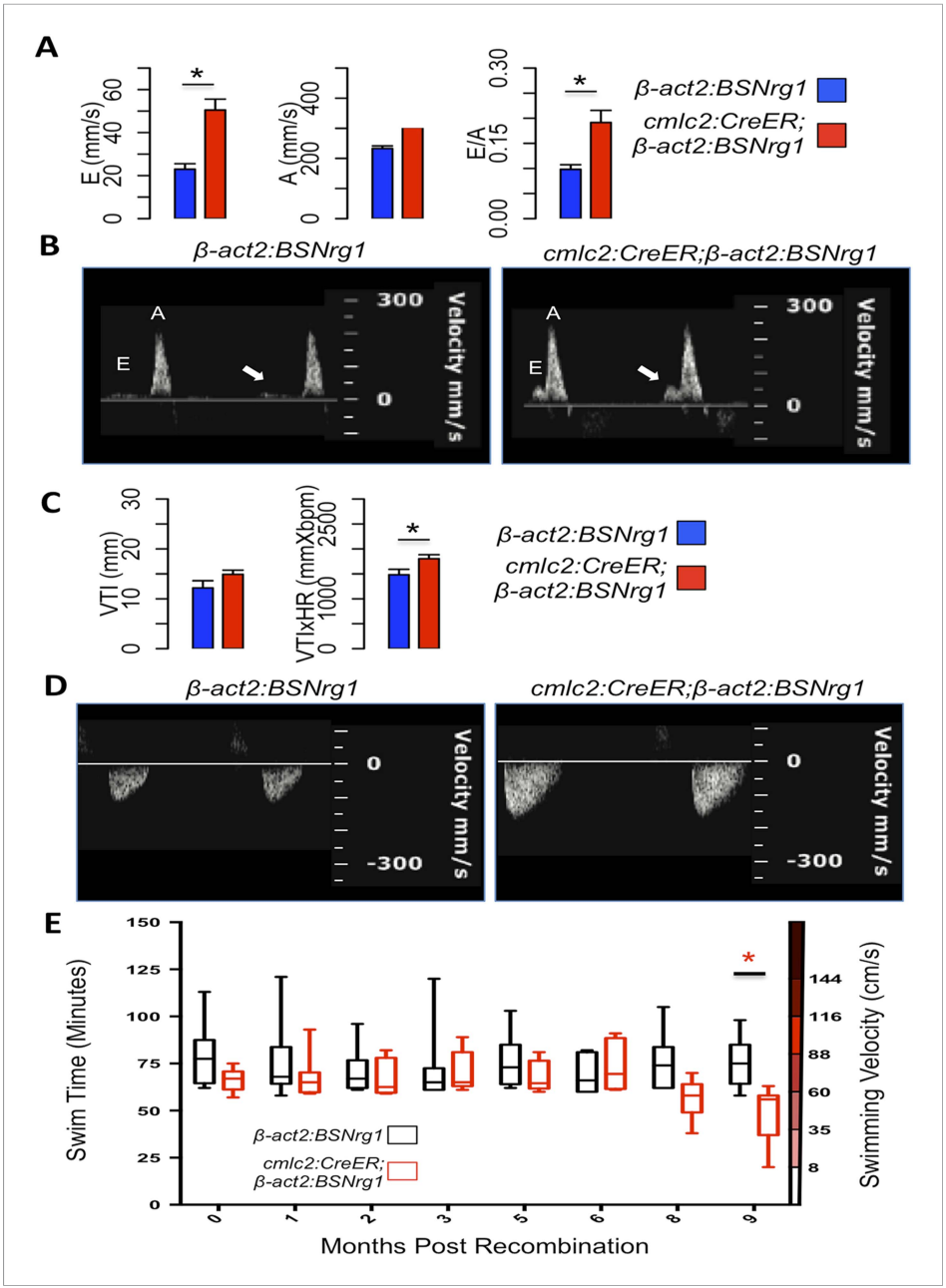

Figure 7. Effects of Nrg1 reactivation on cardiac function. (**A**) Doppler measures of ventricular filling obtained at the AV valve in *cmlc2:CreER; β-act2:BSNrg1* and *β-act2:BSNrg1* animals (n = 9 and 7). Data are represented as mean ± SEM. *p < 0.05, Student's *t*-test, two-tailed. (**B**). Representative PW Doppler at the AV valve in *cmlc2:CreER; β-act2:BSNrg1* and *β-act2:BSNrg1* animals. (**C**) Doppler measures of cardiac output and stroke volume using the velocity time integral (VTI) obtained at the outflow tract (OFT) in *cmlc2:CreER; β-act2:BSNrg1* and *β-act2:BSNrg1* animals (n = 9, 7). Data are represented as mean ± SEM. *p < 0.05, Student's *t*-test, two-tailed. (**D**) Representative PW Doppler at the OFT in *cmlc2:CreER; β-act2:BSNrg1* and *β-act2:BSNrg1* animals. (**E**) Quantification of graded swimming performance of animals at varying times of *nrg1* overexpression plotted as box and whisker plots. Two-way ANOVA was performed looking at the effect of Nrg1 overexpression (p < 0.05), age (p = 0.38), and the interaction of Nrg1 overexpression and age (p < 0.05).

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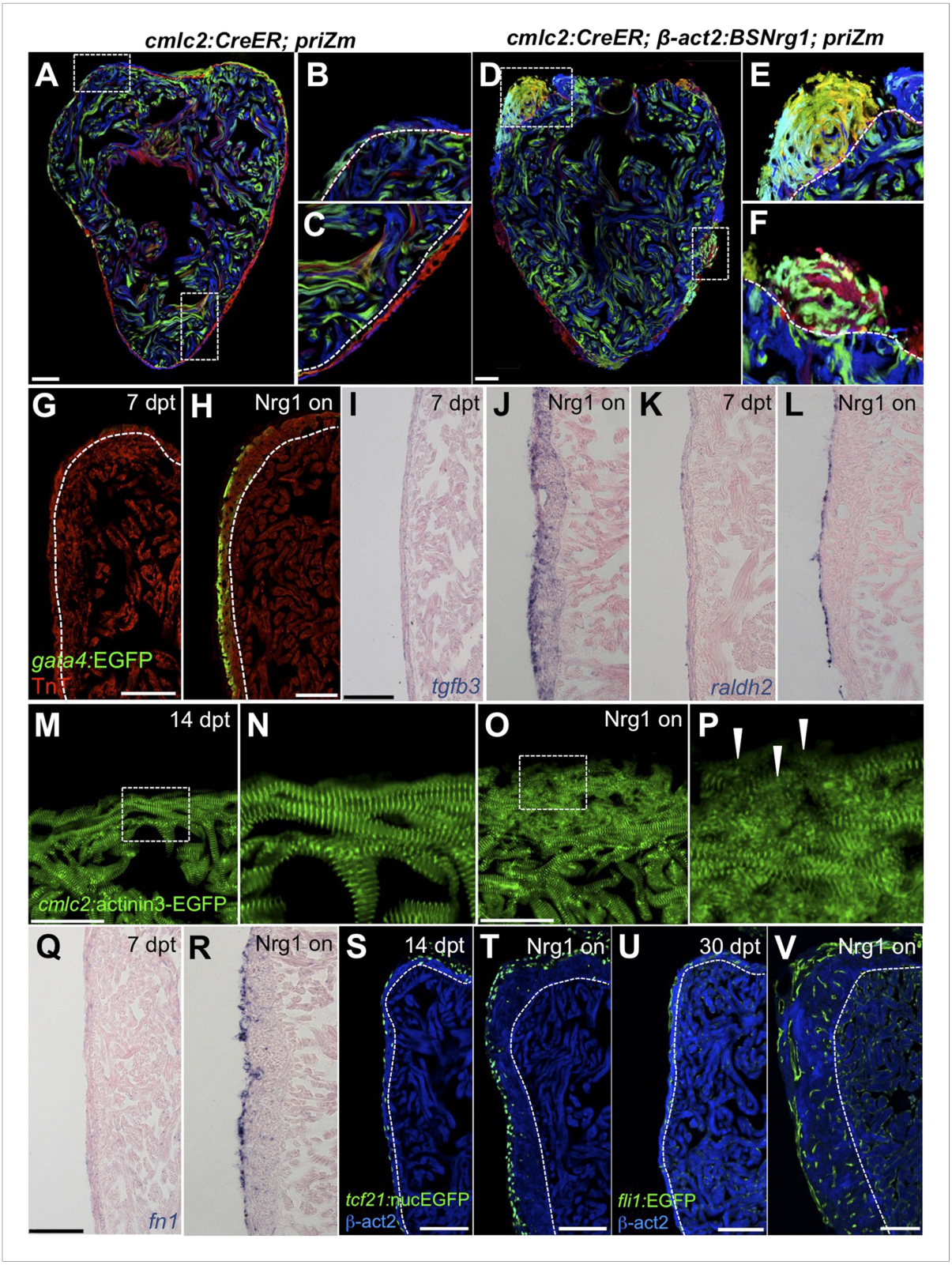

Figure 8. Nrg1 reactivation is sufficient to induce the heart regeneration program. (**A**–**F**) Section images of ventricles from control *cmlc2:CreER; priZM* (**A**–**C**) and *cmlc2:CreER; β-act2:BSNrg1; priZM* (**D**–**F**) animals treated with tamoxifen at 5 weeks post-fertilization (wpf) and assessed at 10 wpf. Cortical myocyte clones show clear boundaries between clones in control ventricles (**B** and **C**; n = 11). During *nrg1* overexpression, cortical muscle thickens appreciably via mixing and radial growth of distinct clones (**E** and **F**; n = 14). Dashed lines delineate cortical from trabecular muscle. Scale bar represents

Figure 8. continued on next page

Figure 8. Continued

100 μm. (**G** and **H**) Section images of ventricles from *cmlc2:CreER; β-act2:BSNrg1* (Nrg1 on) and control animals at 7 days post-treatment, using animals also transgenic for *gata4:EGFP*. EGFP induction is clear in the cortical layer during *nrg1* overexpression. Scale bar represents 100 μm. (**I** and **J**) Section images of ventricles from *cmlc2:CreER; β-act2:BSNrg1* and control animals at 7 days post-treatment, visualized for *tgfβ3* expression by in situ hybridization. Scale bar represents 100 μm. (**K**, **L**, **Q**, **R**) Section images of ventricles from *cmlc2:CreER; β-act2:BSNrg1* and control animals at 7 days post-treatment, visualized for *raldh2* (**K** and **L**) or *fn1* expression (**Q** and **R**) in epicardial cells by in situ hybridization. Scale bar represents 100 μm. (**M** and **N**) Section images of the ventricular wall from *cmlc2:CreER; β-act2:BSNrg1* and control animals at 14 days post-treatment, using animals transgenic for *cmlc2:actinin3-EGFP*. EGFP marks sarcomeric z-bands. Control animals (**M**) show organized sarcomeres in ventricular wall. *nrg1* overexpression (**O**) leads to reduced EGFP fluorescence and disorganization of sarcomeres. Arrowheads point to areas of reduced EGFP intensity and sarcomere organization. Boxes in (**M** and **O**) are represented as high-zoom in (**N** and **P**). Scale bars represents 50 μm. (**S** and **T**) Section images of ventricles from *cmlc2:CreER; β-act2:BSNrg1* and control animals at 14 days post-treatment, visualized for epicardial cells using a *tcf21:nucEGFP* transgene. *nrg1* overexpression grossly increases epicardial cell presence. Scale bar represents 100 μm. (**U** and **V**) Section images of ventricles from *cmlc2:CreER; β-act2:BSNrg1* and control animals at 30 days post-treatment, visualized for endothelial cells using a *fli1:EGFP* transgene. Increased endothelial cells and vasculature are evident in the thickened ventricular wall. Scale bar represents 100 μm.

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