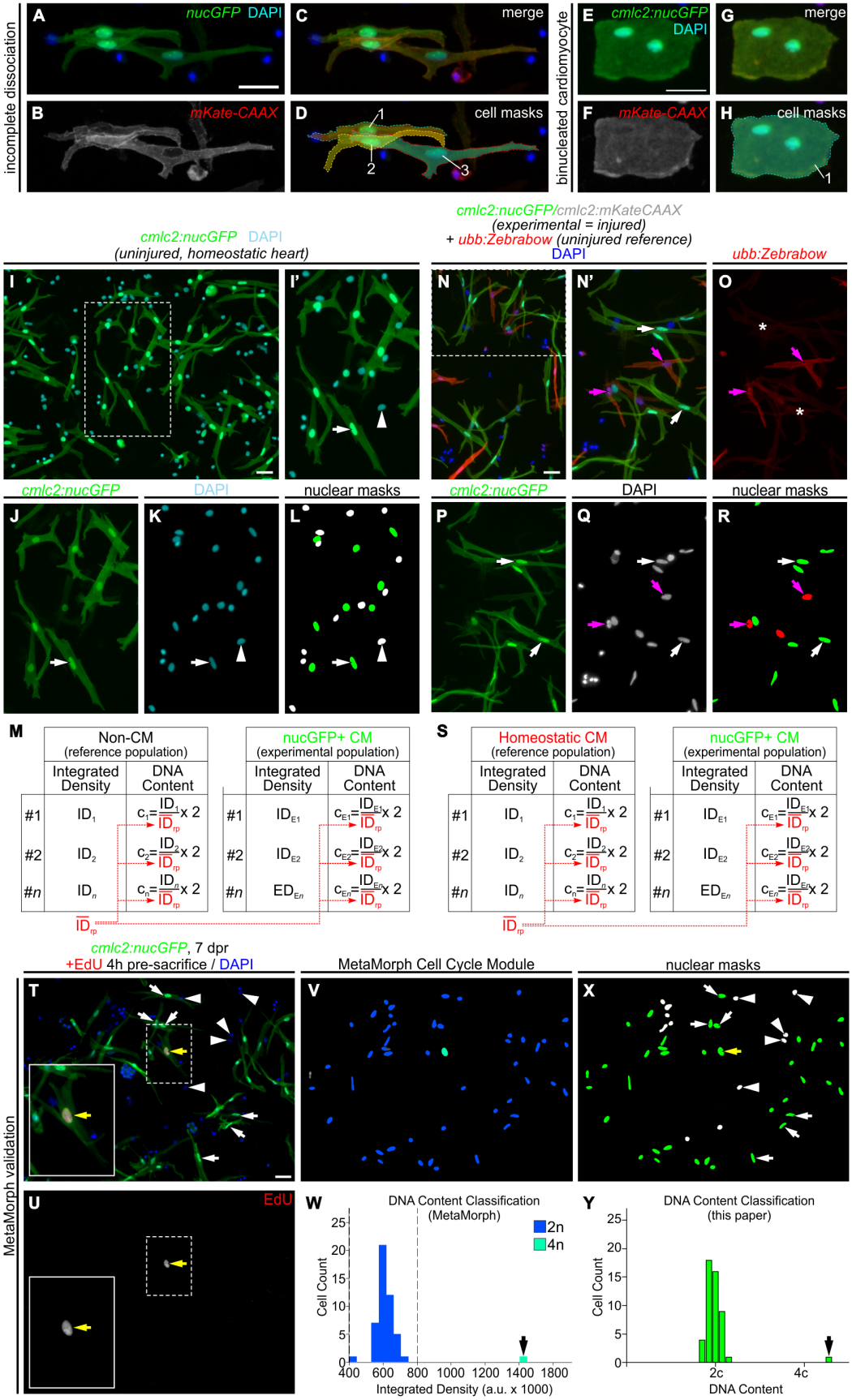


**Figure S1. Quantitative strategies to measure cardiomyocyte nucleation and relative DNA content, Related to Figure 1 and STAR Methods. (A-H) Double transgenic reporters allow myocardial cell**

**aggregates to be distinguished from binucleated cardiomyocytes.** Fluorescent images of cardiomyocytes dissociated from double transgenic *Tg(cmlc2:nucGFP; cmlc2:mKate-CAAX)* hearts. Myocardial cell aggregates (**A-D**) can be clearly distinguished from binucleated cardiomyocytes (**E-H**). **(A,E)** Fluorescent images showing overlapping nuclear GFP and DAPI signals. **(B,F)** Flourescent images showing membrane localized mKate signal. **(C,G)** Merged images of (**A,B**) and (**E,F**), respectively. **(D,H)** Pseudocolored masks based on mKate-CAAX signal highlight cell contours revealing the presence of either three mononucleated cardiomyocytes **(D)** or a binucleated primordial cardiomyocyte **(H)**. **(I-M)** Strategy used to quantify DNA content in cardiomyocytes from homeostatic hearts using non-myocardial cells as the diploid reference population. **(I)** Representative image of cells from dissociated *Tg(cmlc2:nucGFP)* ventricles (as shown in **Figure 1B**), showing GFP and DAPI signals. **(I')** Magnification of boxed area in **(I)**. Cardiomyocytes are GFP+ rod-shaped cells with bright nuclei (white arrow). Non-myocardial cells (i.e. fibroblasts, epicardial and endocardial cells) are GFP- (white arrowhead). **(J,K)** Individual fluorescent channels showing GFP and DAPI signals. **(L)** Nuclear masks were generated based on DAPI signal. Cardiomyocyte and non-cardiomyocyte nuclear masks appear in green and white, respectively. **(M)** For each nucleus, the integrated density (ID) of the DAPI signal was obtained. The average ID value of non-cardiomyocytes (reference population, $ID_{rp}$, red) was determined for every picture. This value represents the average DNA content of the diploid population (2c). Then, the ID value of each cell from both populations was divided by the $ID_{rp}$ and multiplied by 2. The resulting value represents the DNA content of a given cell relative to the reference population. See STAR Methods section for further details. **(N-S)** Strategy used to quantify DNA content in cardiomyocytes from regenerating hearts using homeostatic cardiomyocytes as the reference diploid population. Double transgenic *Tg(cmlc2:nucGFP; cmlc2:mKate-CAAX)* animals were subjected to apical resection. Because polyploidization of non-myocardial cells from amputated hearts cannot be ruled out, injured ventricles were dissociated in combination with uninjured ventricles from *Tg(ubb:Zebrabow)* animals, that express mCherry in all cells. mCherry+ *ubb:Zebrabow* cardiomyocytes were used as the reference diploid population. **(N)** Representative image of cells from dissociated injured *Tg(cmlc2:nucGFP; cmlc2:mKate-CAAX)* and uninjured *Tg(ubb:Zebrabow)* ventricles, showing mCherry, GFP and DAPI signals. **(N')** Magnification of boxed area in **(N)**. Cardiomyocytes from regenerating hearts are nucGFP+ (white arrows) and cardiomyocytes from homeostatic hearts are mCherry+ (pink arrows). **(O-Q)** Individual fluorescent channels showing mCherry, GFP, and DAPI signals. Asterisks in **(O)** indicate faint signal from mKate-CAAX in regenerating cardiomyocytes. **(R)** Nuclear masks were generated based on DAPI signal. Regenerating cardiomyocyte and homeostatic cardiomyocyte nuclear masks appear in green and red, respectively. **(S)** For each nucleus, the ID of the DAPI signal was obtained. The average ID value of the mCherry+ reference population ($ID_{rp}$, red) was determined for every picture. This value represents the average DNA content of the diploid population (2c). Then, the ID value of each cell was divided by the $ID_{rp}$ and multiplied by 2. The resulting value represents the DNA content of a given cell relative to the reference diploid population. **(T-Y)** Validation of our quantification strategy using the MetaMorph Cell Cycle Module. **(T)**

Representative image of dissociated cells from *Tg(cmlc2:nucGFP)* ventricles at 7 days post-resection. Animals were injected with 10 $\mu$M EdU 4 hours before dissociation to label proliferating cells. **(U)** Individual fluorescent channels showing EdU signal. Boxed areas are shown at higher magnification. Yellow arrow indicates an EdU+ cardiomyocyte nucleus. **(V-W)** Ploidy measurement of image shown in **(T)** using MetaMorph software. **(V)** Color-coded masks automatically generated by the MetaMorph Cell Cycle Module based on DAPI integrated density. **(W)** DNA content histogram generated by MetaMorph. The software identified a cell with double the DNA content compared to the rest of the population (black arrow). **(X,Y)** Ploidy measurement of image shown in **(T)** using the quantification strategy described above. **(X)** Nuclear masks generated manually based on DAPI signal. Cardiomyocyte and non-cardiomyocyte nuclear masks appear in green and white, respectively. **(Y)** DNA content histogram generated by the quantification strategy described above. This method identified a cell with double the DNA content compared to the rest of the population (black arrow). Scale bars: 50 $\mu$m.

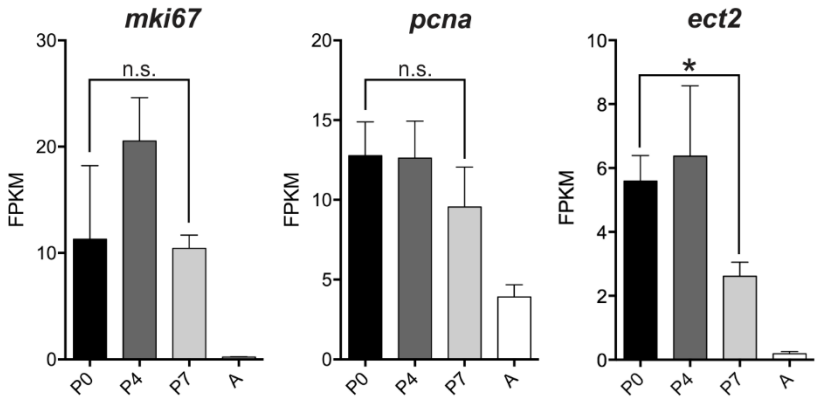


**Figure S2. Ect2 is downregulated during mouse cardiomyocyte maturation, Related to Figure 1.** Data extracted from publically available sources (O'Meara et al., 2015). *Ect2* declines at the onset of cardiomyocyte polyploidization (P7) while other cell cycle-related transcripts (*mki67, pcna*) remain unchanged. Samples were obtained from whole ventricles. *, $p<0.05$, unpaired *t*-test. A, adult; P, postnatal day.

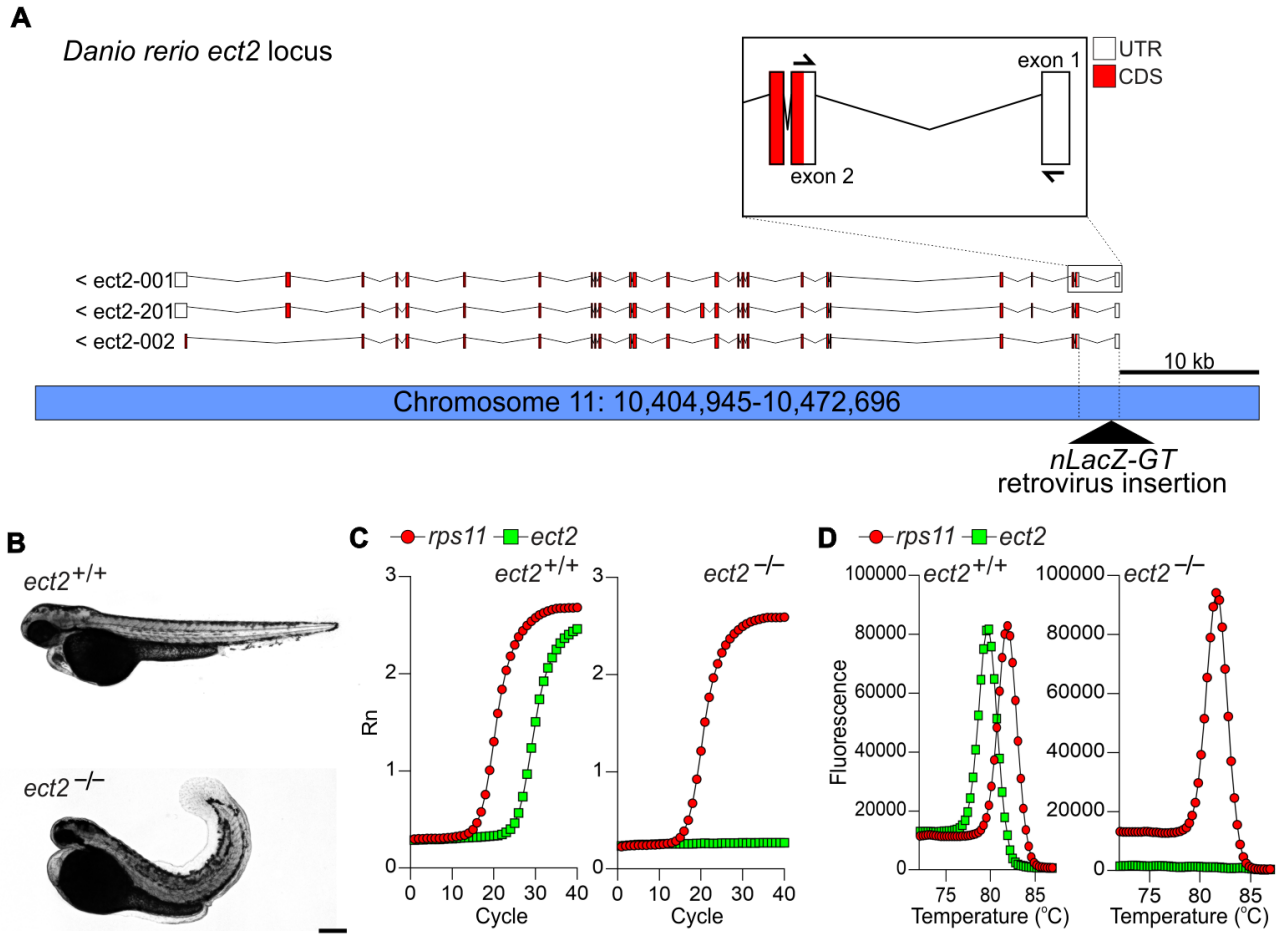


**Figure S3. The retroviral insertion *hi3820aTg* results in disrupted splicing of *ect2* mRNA, Related to Figure 2. (A)** Schematic representation of the *Danio rerio ect2* genomic locus and transcript variants. The *hi3820aTg (nLacZ-GT)* retroviral integration site is located in the first intron. The retroviral sequence includes splice acceptor sequence and the *nLacZ* cDNA. Boxed area shows at higher magnification the first three exons and the location of real-time PCR primers used to detect correct splicing of *ect2* mRNA. **(B)** Bright-field images of control (*ect2*$^{+/+}$) and *ect2*$^{hi3820aTg/\ hi3820aTg}$ *(ect2*$^{-/-}$*)* embryos at 72 hpf. Mutant embryos exhibit smaller heads and curly tails. **(C,D)** Real-time PCR amplification (**C**) and dissociation **(D)** curves of *rps11* (housekeeping control) and *ect2* in ect2$^{+/+}$ and *ect2*$^{-/-}$ embryos, revealing splicing disruption in *ect2* mutant embryos. Scale bar: 200 μm.

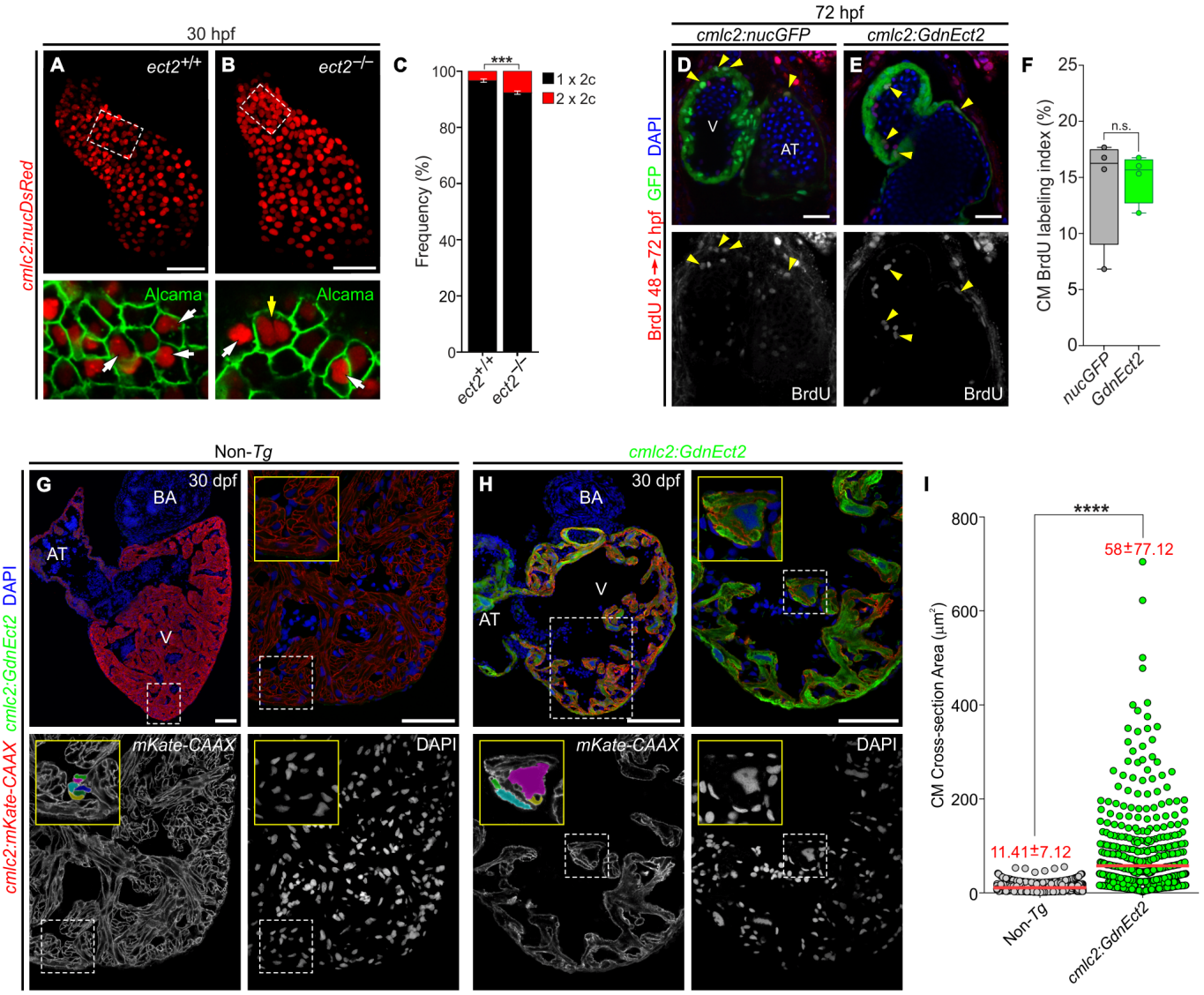


**Figure S4. Ect2 is required for cardiomyocyte cytokinesis, but dispensable for entry into S-phase, and constitutive myocardial *dnEct2* expression induces cardiomyocyte hypertrophy, Related to Figure 2. (A,B)** Confocal projections of embryonic hearts of *ect2*$^{+/+}$ (**A**) and *ect2*$^{-/-}$ (**B**) animals carrying the *Tg(cmlc2:nucDsRed)* transgene at 30 hpf. Single confocal planes of boxed regions are shown at higher magnification with Alcama immunostaining to highlight plasma membranes. White and yellow arrows point to diploid and polyploid cardiomyocytes, respectively. **(C)** Quantification of indicated cardiomyocyte populations from ect2$^{+/+}$ (n=4) and ect2$^{-/-}$ (n=4) hearts at 30 hpf (mean±s.d; ** P<0.01 by two-tailed unpaired *t*-test). **(D,E)** Single confocal planes of 72 hpf *Tg(cmlc2:nucGFP)* (**D**) and *Tg(cmlc2:GdnEct2)* (**E**) animals exposed to BrdU between 48 and 72 hpf, immunostained for GFP and BrdU and counterstained with DAPI. Yellow arrowheads indicate BrdU+ cardiomyocyte nuclei. The BrdU signals from (**D**) and (**E**) are shown below. **(F)** Quantification of the *Tg(cmlc2:nucGFP)* (n=4) and *Tg(cmlc2:GdnEct2)* (n=4) cardiomyocyte BrdU labeling index at 72 hpf (mean ± s.d, non-significant by two-tailed unpaired t-test). **(G,H)** Ventricular sections from 30 dpf non-*Tg* **(G)** or *Tg(cmlc2:GdnEct2)* animals **(H)** carrying the *Tg(cmlc2:mKate-CAAX)* transgene to label cardiomyocyte cell membranes. Hearts were immunostained to detect mKate and GFP and

counterstained with DAPI. Boxed regions are shown at higher magnifications (right and insets). Individual fluorescent channels showing mKate and DAPI signals (bottom) as indicated. Pseudocolored cells depicting the cross-sectional area of individual cardiomyocytes. **(I)** Quantification of cardiomyocyte cross-sectional area in non-*Tg* (n=1996 total cardiomyocytes from 3 animals) and *cmlc2:GdnEct2* (n=688 total cardiomyocytes from 5 animals). Mean values and standard deviations are shown in red. ****, $P<1\cdot10^{-15}$, Mann-Whitney test. Scale bars: 25 μm (A-B, D-E) 100 μm (G-H), 50 μm (magnifications from G and H).

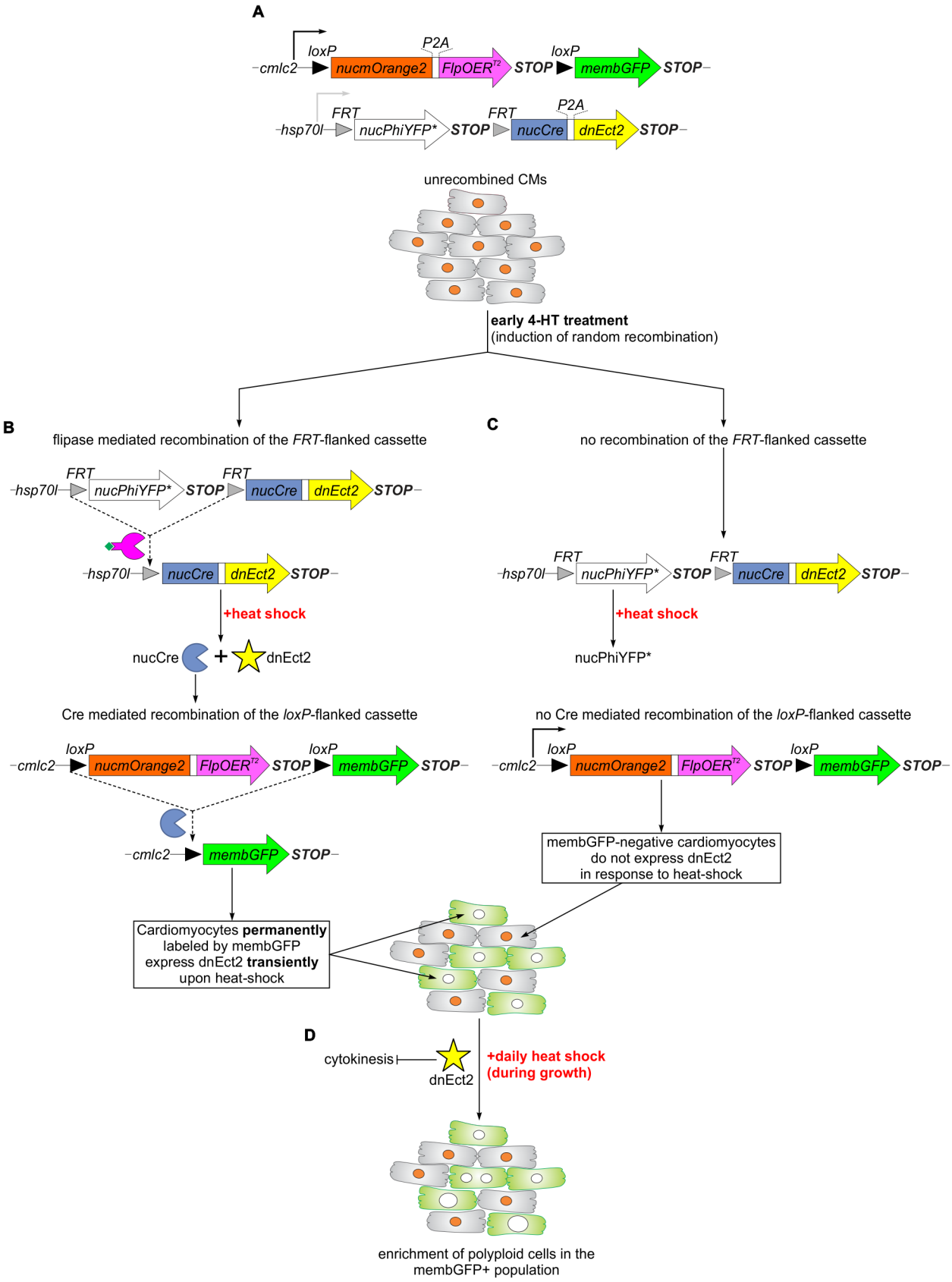


**Figure S5. Transgenic strategy used to create mosaic zebrafish hearts composed of GFP- diploid and GFP+ polyploid-enriched cardiomyocyte populations, Related to Figure 3, Figure 4 and STAR Methods. (A)** In untreated hearts, the *cmlc2:(iF-S)$^{lox}$-mG* transgene (top) directs the expression of a *loxP*-

flanked cassette that contains a nuclear-localized mOrange2 fluorescent protein and a tamoxifen-inducible Flipase (FlpOER$^{T2}$). The *hsp:(P*-S)$^{FRT}$-Cre-dnEct2* transgene (bottom), which only becomes activated in response to heat-shock, directs expression of a *FRT*-flanked cassette that contains a mutated, non-fluorescent nuclear-localized PhiYFP protein (nucPhiYFP*, abbreviated *P**). The presence of the *loxP*- and *FRT*-flanked cassettes prevents the expression of downstream genes. Therefore, untreated animals carrying both transgenes express nuc-mOrange2 and FlpOER$^{T2}$ specifically in cardiomyocytes. Heat-shock treatment induces the expression of nucPhiYFP*. **(B,C)** Exposure to 4-HT during development induces FlpOER$^{T2}$-mediated recombination of the *FRT*-flanked cassette (in the *hsp:(P*-S)$^{FRT}$-Cre-dnEct2* transgene) randomly in some cardiomyocytes. **(B)** Cardiomyocytes that undergo FlpOER$^{T2}$-mediated recombination and are exposed to heat-shock express Cre and dnEct2. As a consequence, Cre catalyzes the recombination of the *loxP*-flanked cassette in the *cmlc2:(iF-S)$^{lox}$-mG* transgene resulting in cardiomyocytes that are permanently labeled by GFP. Heat-shock treatment induces expression of Cre and dnEct2. **(C)** Cardiomyocytes that do not undergo FlpOER$^{T2}$-mediated recombination and are exposed to heat-shock express nucPhiYFP* but do not express Cre and dnEct2. In the absence of Cre, the *cmlc2:(iF-S)$^{lox}$-mG* transgene remains unrecombined and cardiomyocytes are not labeled by GFP. GFP- cardiomyocytes do not express Cre or dnEct2 in response to heat-shock. This binary system creates mosaic hearts containing GFP- and GFP+ populations, the latter being susceptible to polyploidization through dnEct2-mediated cytokinesis inhibition. **(D)** Embryos containing mosaic hearts are initially grown in the absence of heat-shock to allow the expansion of the GFP+ and GFP- populations. When animals are 2-3 weeks old, they are exposed to daily heat-shocks for the following 3-4 months. Heat-shock induces the expression of dnEct2 in the GFP+ population, inhibiting cytokinesis. Consequently, the GFP+ population becomes enriched in polyploid cells while the GFP- population remains diploid.

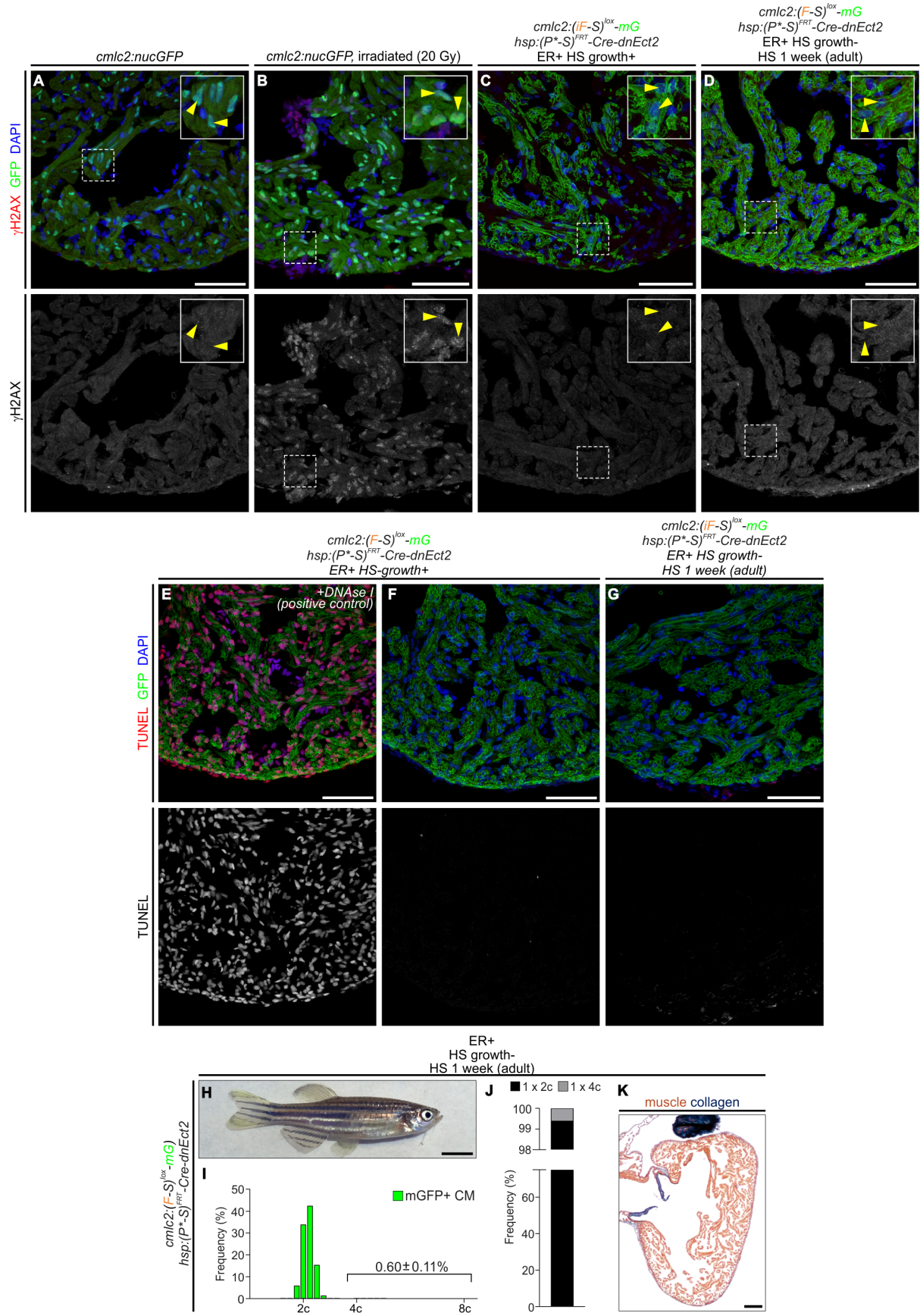


**Figure S6. Myocardial *dnEct2* expression does not induce DNA damage or apoptosis, and exposure to dnEct2 during adulthood does not induce cardiomyocyte polyploidization, Related to Figure 3**

**and Figure 4. (A-D)** Sections from hearts from the indicated cohorts, immunostained for γH2AX (to detect DNA damage), GFP and counterstained with DAPI. γH2AX signals are shown in bottom panels. Boxed regions are shown at higher magnifications (insets). Irradiated animals (**B**) serve as a positive control for the DNA damage response. Yellow arrowheads in insets indicate cardiomyocyte nuclei. **(E-G)** TUNEL staining in sections from hearts from the indicated cohorts, immunostained for GFP and counterstained with DAPI. TUNEL signal is shown in bottom panels. Section in (**E**) was incubated in DNAse I as a positive control for apoptosis. **(H)** External appearance of double-transgenic adult zebrafish, subjected to early recombination during embryogenesis, grown in the absence of polyploid-inducing heat-shock treatments and exposed to one week of daily heat-shock treatments during adulthood. **(I,J)** DNA content (**I**) and quantification of indicated cardiomyocyte populations **(J)** from (**H**). n=2839 total cardiomyocytes from 3 replicates, 3 ventricles per replicate. Scale bars: 50 μm (A-G, K), 5 mm (H).

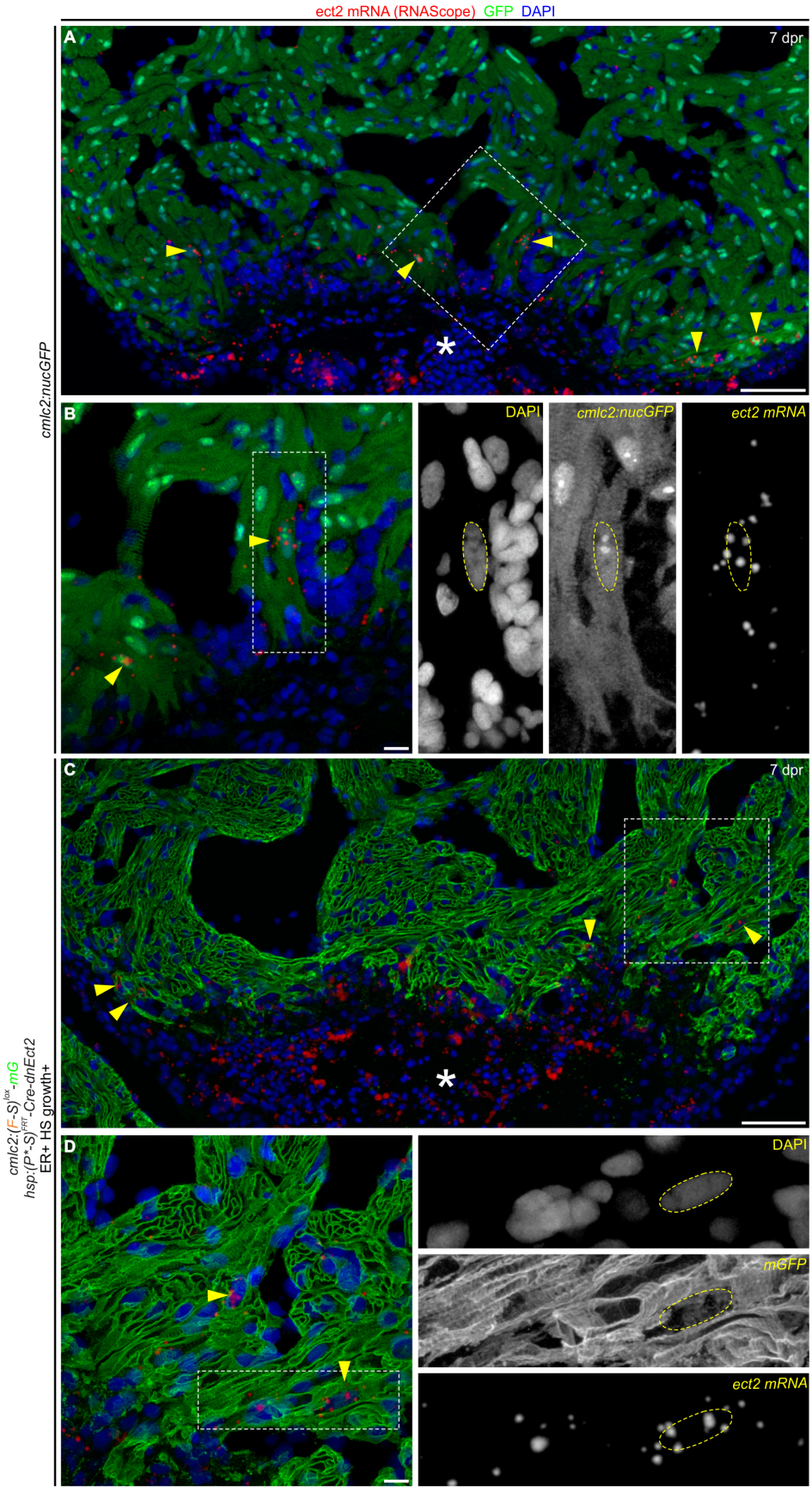


**Figure S7. *Ect2* is re-expressed after injury in polyploid-enriched hearts, related to Figure 5 and Figure 6. (A,C)** RNAScope in situ hybridization showing *ect2* transcripts (yellow arrowheads) in a section

from the indicated cohorts at 7 dpr, immunostained for GFP and counterstained with DAPI. Asterisk indicates the wound area. **(B,D)** Magnified regions from (**A**) and (**D**). Boxed region is shown to the right with individual fluorescent signals for DAPI, GFP and *ect2* mRNA. n=4 hearts per group with 3 sections per heart. Scale bars: 50 μm (A,C), 10 μm (B,D).

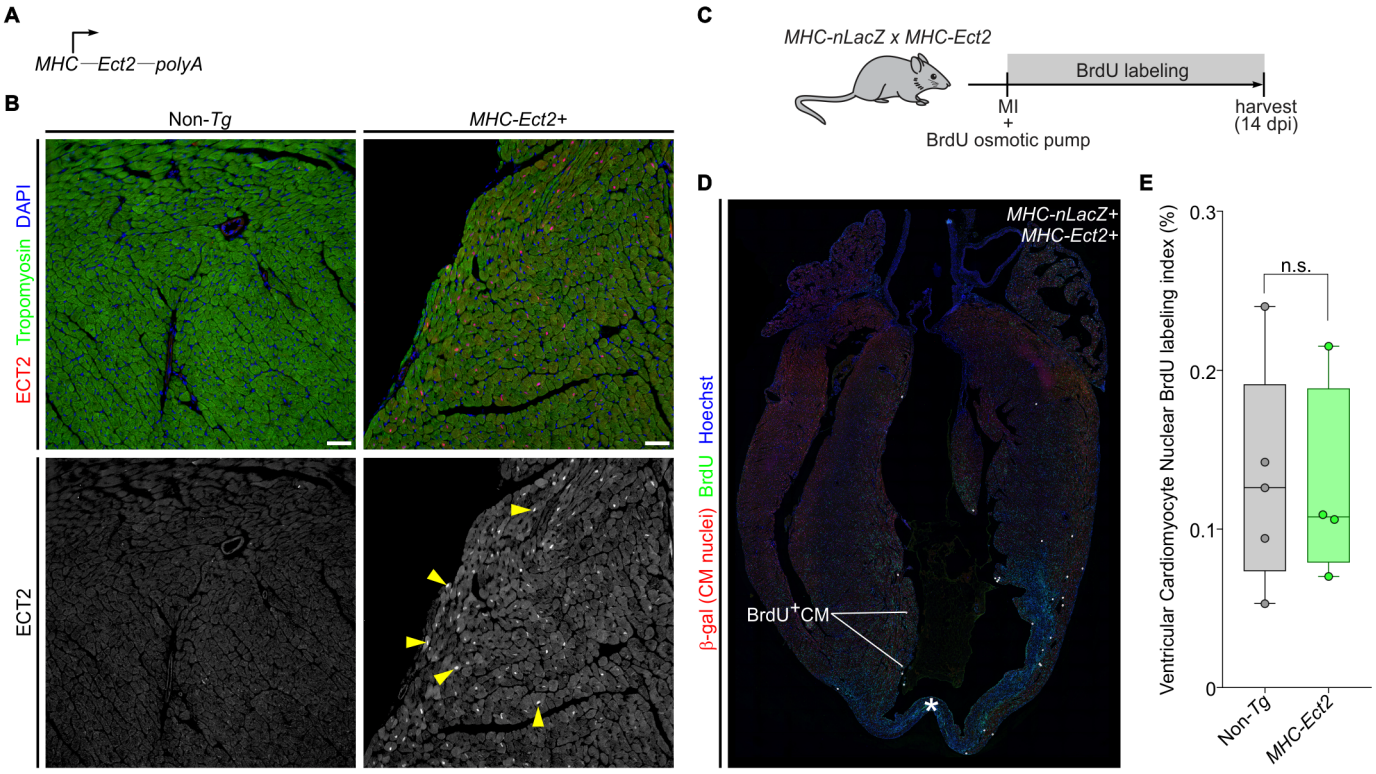


**Figure S8. Constitutive Ect2 expression in the myocardium of mouse hearts is insufficient to increase cardiomyocyte proliferation after injury, Related to Figure 5 and 6. (A)** Transgene used to overexpress murine Ect2 in mouse cardiomyocytes. **(B)** Sections from ventricles from non-*Tg* and *MHC-Ect2* transgenic siblings, immunostained for Ect2, Tropomyosin and counterstained with Hoechst (top). Ect2 signal is shown in bottom panels. Yellow arrowheads indicate Ect2+ cardiomyocyte nuclei. **(C)** Experimental strategy used to evaluate cardiomyocyte proliferation after myocardial infarction (MI) in mouse. All mice carried the *MHC-nLacZ* transgene to identify cardiomyocyte nuclei. **(D)** Representative section of a *MHC-nLacZ+*, *MHC-Ect2+* animal at 14 days post-infarction, immunostained for β-galactosidase, BrdU and counterstained with DAPI. White circles highlight BrdU+ ventricular cardiomyocyte nuclei. **(E)** Ventricular cardiomyocyte BrdU labeling indices in non-Tg (n=116,457 cardiomyocytes scored from 5 ventricles, 2 sections per ventricle) and MHC-Ect2+ (n=122,571 cardiomyocytes scored from 4 ventricles, 2 sections per ventricle) animals. Scale bar: 50 μm.