Supplementary Fig S1. FUCCI-Tg expression is exclusive to cardiomyocytes and not in any non-myocyte lineages. FUCCI-Tg cardiomyocytes, AzG (green) or mKO (red), do not colocalize with immunohistochemistry labeling of smooth muscle 22α (Sm22α, smooth muscle lineage, magenta) (A), von Willebrand factor (vWF, endothelial lineage, magenta) (B), and Vimentin (Vim, fibroblast lineage, magenta) (C). Cardiomyocytes lineages are confirmed by Tropomyosin (TPM, blue, A-B) or Cardiac Troponin I (cTnI, blue, C), nuclei in white (Sytox, A-B; DAPI, C), scale bar 20μm. Cultured Cardiac Progenitor Cells (CPCs) are AzG and mKO negative (D, D') but show low expression of ubiquitin Geminin (cyan, D'') and αMHC promoter (magenta, D'''), Scale bar 50μm. (E) Immunoblot of αMHC and FUCCI-Tg constructs in whole heart lysate, isolated non-myocytes, and cultured CPCs. αMHC, fusion protein AzG-Geminin and mKO-Cdt1 are high in whole heart, low in non-myocytes, and low to undetectable in CPCs. Hsp60 was used as loading control.

Supplementary Fig S2. Isolated P2 FUCCI cardiomyocytes in culture allow visualization of cell division and binucleation events. Isolated neonatal cardiomyocytes from FUCCI hearts show progression from S/G2/M (green arrow, A) into a cleavage furrow (white arrow, B) ending in two distinct daughter cells (red arrows, C). Neonatal cardiomyocyte culture allows visualization and progression from G1 (red arrow, D), into G1/S (orange arrow, E) and S/G2/M (green arrow, F) before ending in a binucleation event (red arrows, G). Native fluorescence shows AzG (green) and mKO (red) in brightfield snapshots of Movie S1 and Movie S2, scale bar 50μm.

Supplementary Fig S3. Adult FUCCI-Tg CMs regulate FUCCI probes accordingly throughout development. Representative immunoblot shows increased accumulation of ubiquitinated protein in adult FUCCI ACMs after MG132 treatment (10μM) in (A) and quantified in (B); ***P<0.0001 vs DMSO Ctrl. Representative immunoblot of AzG-Gem and mKO-Cdt1 accumulation after MG132 proteasome block as in (C). Quantitation of AzG; ***P<0.0001 (D, left) and mKO; NS (D, right) vs DMSO treated samples. N=3-5 mice per time point analyzed, n=4055, 1953, 7536, 2861, 4765, 3351 nuclei for P0, P2, P7, P14, P21, P90 (A, B). N=5-6 individual CM isolations (A, C).

Supplementary Fig S4. FVB non-transgenic hearts show increasing levels of endogenous Geminin and Cdt1. Immunoblot of P2-P30 FVB/NJ whole heart lysates (A). Quantitated levels of endogenous geminin (B, left) and Cdt1 (B, right) protein expression from A. (C) Isolated P90 FVB/NJ cardiomyocytes immunolabeled for endogenous Geminin (green, C), DAPI (blue, D), and αSA (red, E; merged), scale bar 20μm. N=3 hearts per time point, *P<0.05, **P<0.001, ***P<0.0001 vs P2, unpaired t-test.

Supplementary Fig S5. Border zone cardiomyocytes fail to incorporate BrdU through 10dpi. Representative merged images of IZ/BZ showing BrdU incorporation in interstitial population, not cardiomyocytes at 3 (A), 7 (B) and 10dpi (C) respectively. Native fluorescence shows AzG (green, A'-C'), mKO (red, A'-C'), immunostaining shows BrdU (magenta, A'-C'), Sytox (white, A"-C"), and cTnI (blue, A"-C"). Scale bar 50μm. N= 3-4 hearts per time point.

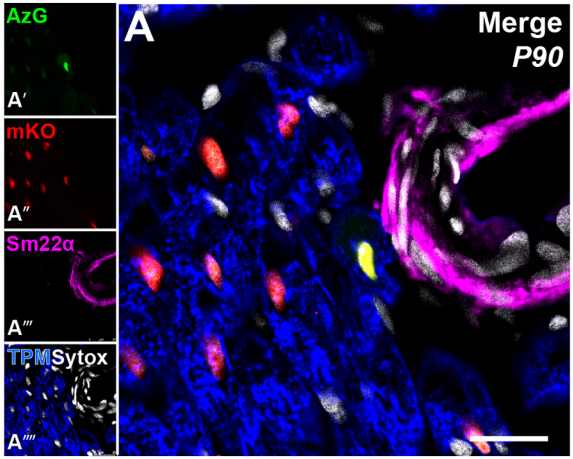
Supplementary Fig S6. Fig. S6. Border zone cardiomyocytes exhibit limited BrdU integration at 21dpi. Representative images of sporadic IZ/BZ CMs showing BrdU incorporation $BrdU^+$at G1/S (AzG^+/mKO^+) (A), $BrdU^+$at G1 (AzG^-/mKO^+) (B). Native fluorescence shows AzG (green, A', B'), mKO (red, A', B'), immunostaining shows BrdU (cyan, A", B"), Sytox (white A"', B"'), and cTnI (blue, A'", B'"). Scale bar 20µm. (C) Quantification of percent BrdU+CM of all CM at various cell cycle arrest at 21dpi. *P<0.05. N=4 hearts, n=149 tissue sections, and n=6705 individual nuclei. One-way ANOVA, Dunnett's post hoc test.

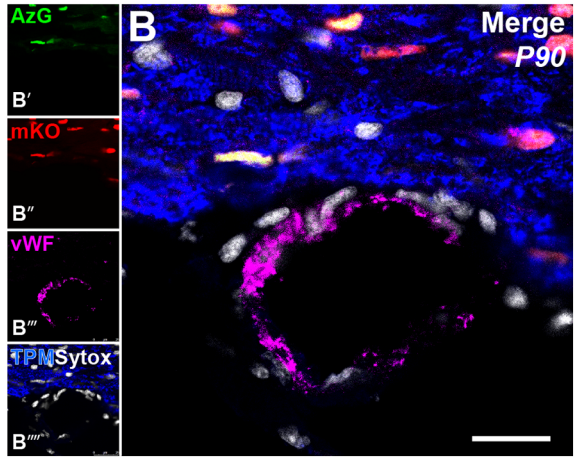
Supplementary Fig S7. Remote Zone FUCCI cardiomyocytes fail to incorporate BrdU through 21dpi. Representative final merged images of remote zone cardiomyocytes from 3 (A), 7 (B), 10 (C), 14 (D) and 21dpi (E) tissue sections show lack of BrdU incorporation in cardiomyocytes. Native fluorescence for AzG (green, A'-E'), mKO (red, A'-E') immunofluorescence for BrdU (magenta, A"-E"), Sytox (white, A-E) and cTnI (blue, A-E). Percent cardiomyocyte nuclei at G0; ns, G1; ###P<0.0001 vs. P90. S/G2/M, **P<0.001, ***P<0.0001 vs. P90 (F) and G1/S interface; **P<0.001, ***P<0.0001 vs. P90. (G). Scale bar 50µm, N= 3-4 hearts per time point. One-way ANOVA, Tukey's post hoc test.

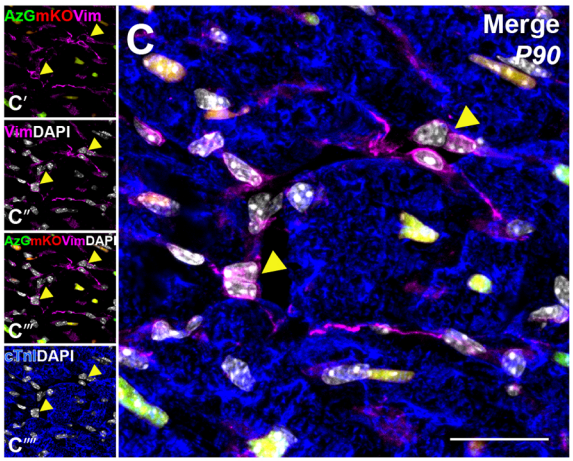
Supplementary Fig S8. Isoproterenol induced cardiotoxic injury fails to force cardiomyocyte cell cycle re-entry through 28dpi. Schematic of Isoproterenol (single dose, 150mg/kg) injury and daily BrdU (50mg/kg) pulse (A) Representative merged confocal images of isoproterenol induced cardiotoxic injury at 3 and 7dpi (B, C). Native fluorescence for AzG (green, B', C'), mKO (red, B', C') and immunolabeling of BrdU (magenta, B", C"), cKit (cyan, B", C"), Sytox (white, B, C), and cTnI (blue, B, C). (D) Percent cardiomyocyte nuclei at G0: *P<0.05 vs. P90, G1; #P<0.05 vs. P90; S/G2/M: $P<0.05 vs. P90. (E) Percent cardiomyocyte nuclei at G1/S; ns. n= 3351 (P90), 1707 (3dpi), 1344 (7dpi), 1027 (14dpi), 839 (21dpi), 937 (28dpi) nuclei counted utilizing 9 sections from 3-4 hearts per time point. One-way ANOVA, Tukey's post hoc test.

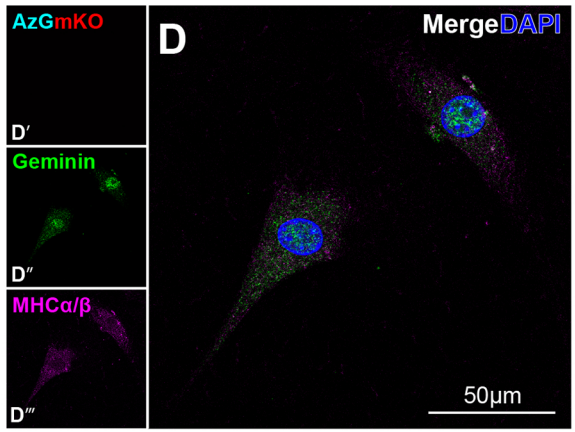
Supplementary Video S1. P2 cardiomyocyte cell division. Time-lapse video of isolated FUCCI-Tg P2 cardiomyocyte for 72 hours in culture. One dividing AzG^+ CM (green) gradually lost AzG expression, displayed midbody furrow (white arrow), and divided into two separated daughter cells (red arrows). Scale bar 75µm.

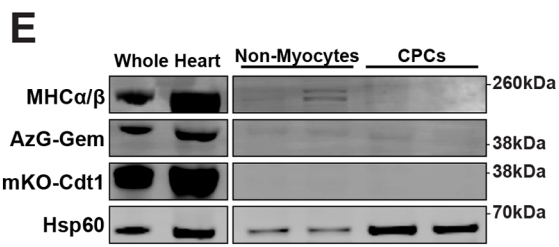
Supplementary Video S1. P2 cardiomyocyte binucleation. Time-lapse video of isolated FUCCI-Tg P2 cardiomyocytes for 64 hours in culture. One CM progressed through cell cycle from G1 (mKO^+, red, red arrow), S (mKO^+/AzG^+, orange to yellow, orange arrow), S/G2/M (AzG^+, green, green arrow). Nuclear $AzG^+$was gradually lost before a single nucleus became two colorless nuclei (G0/G1). Scale bar 75µm.





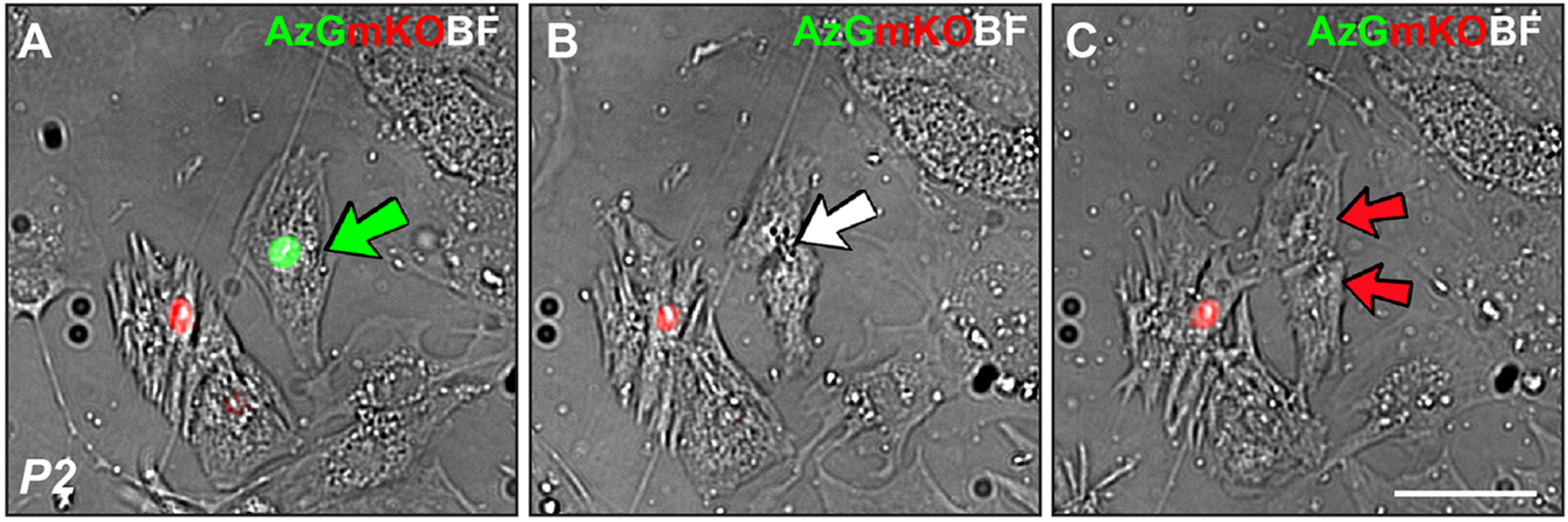




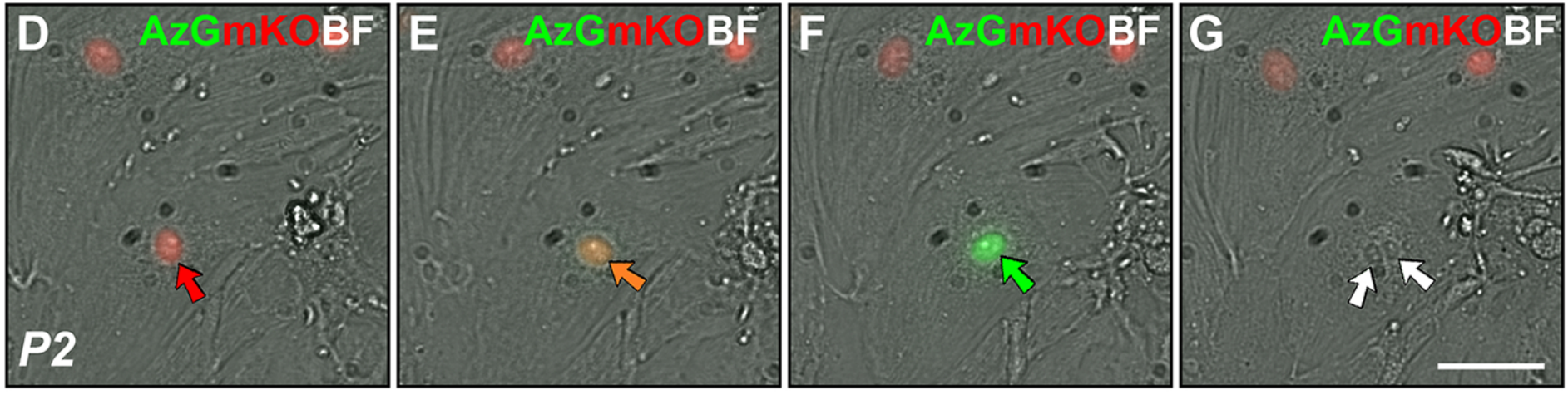


Supplementary Fig S1

Cell Division



Binucleation



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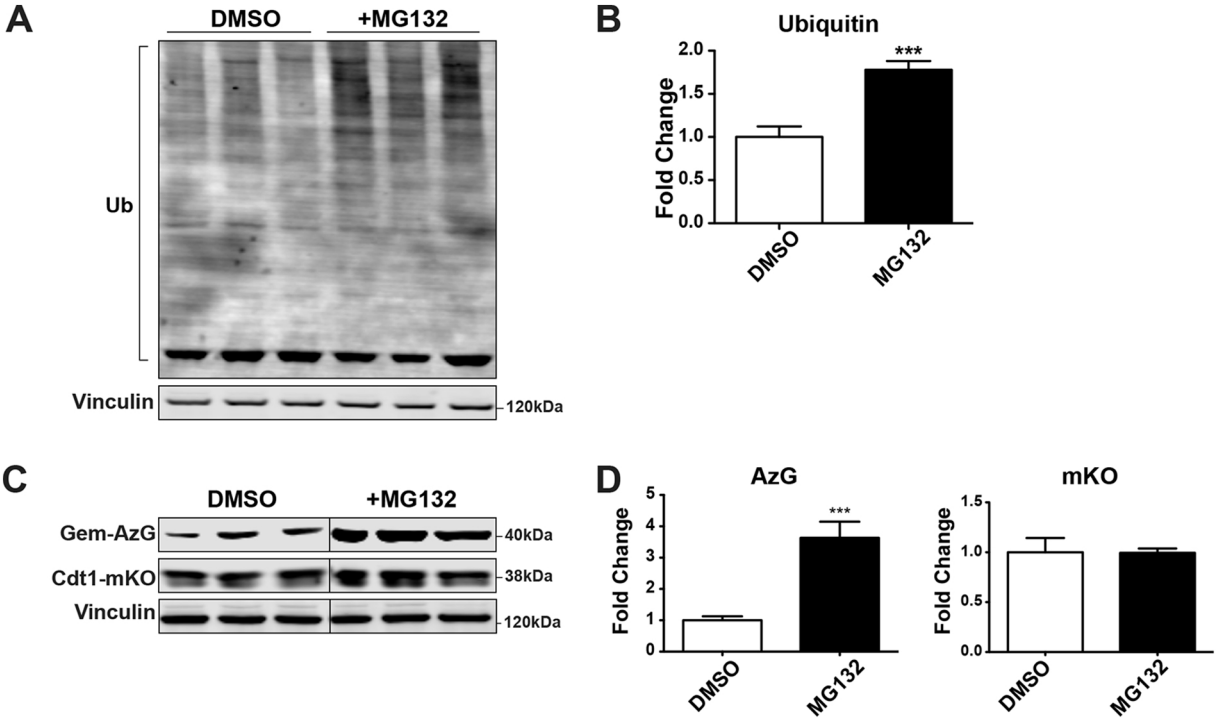

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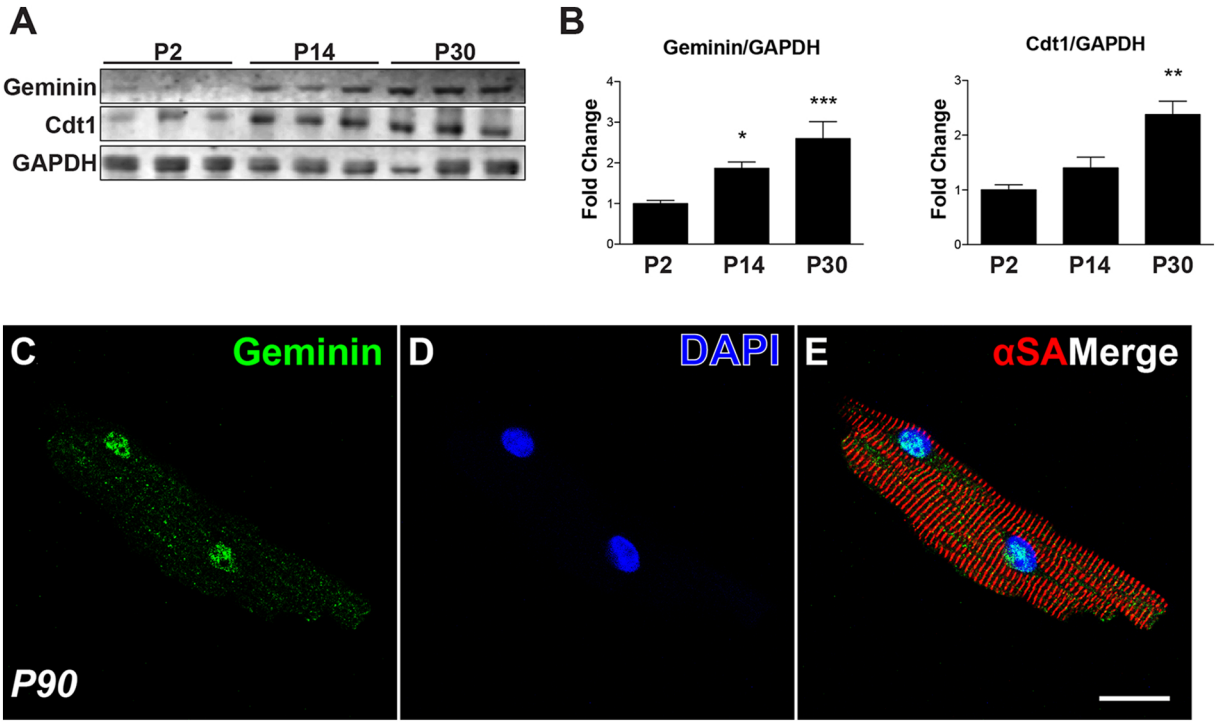

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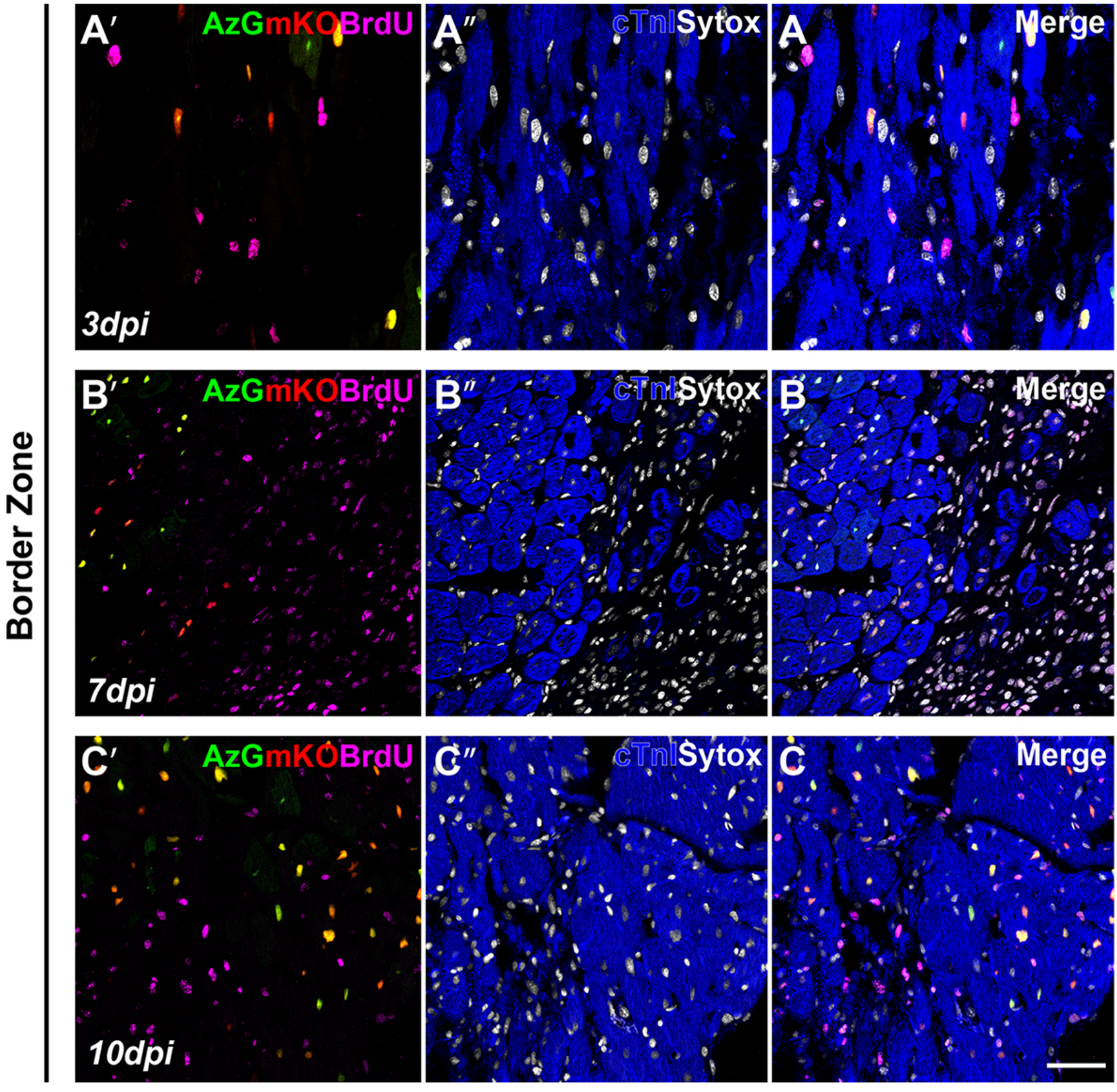

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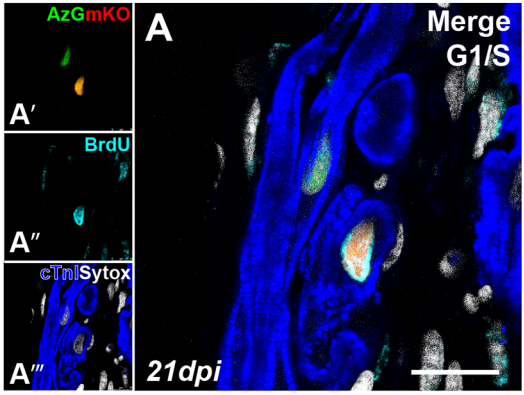

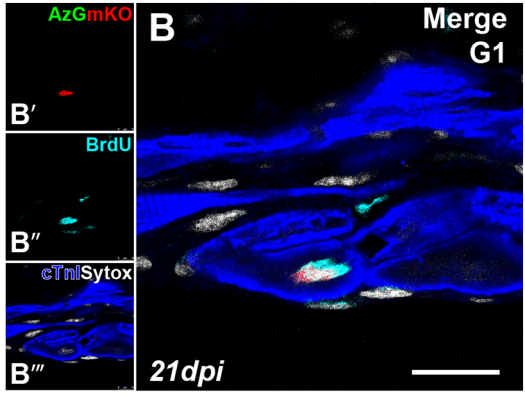

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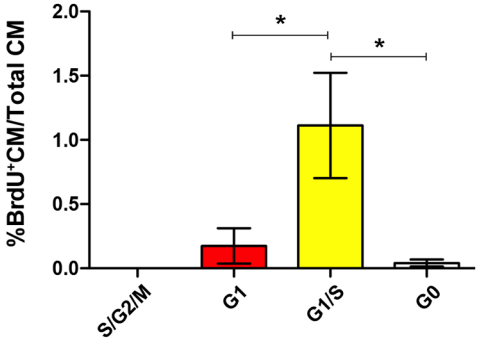

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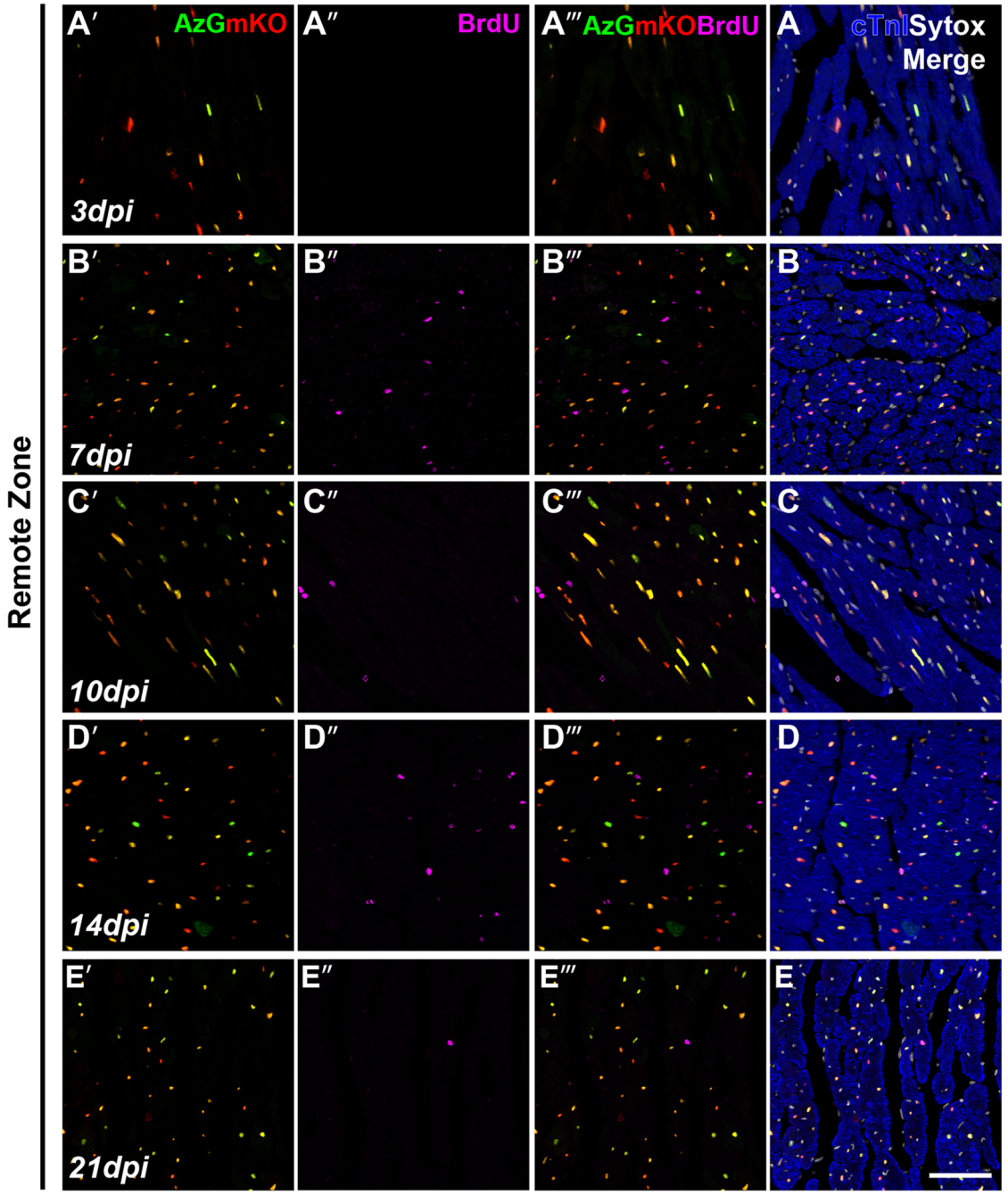

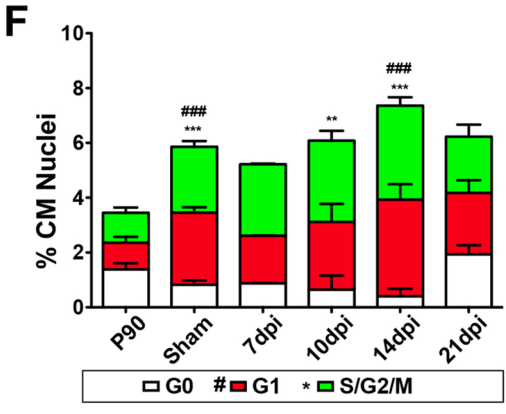

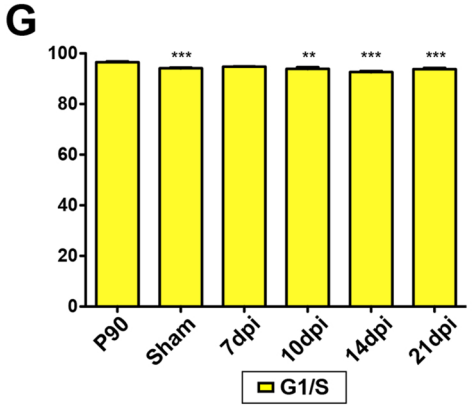

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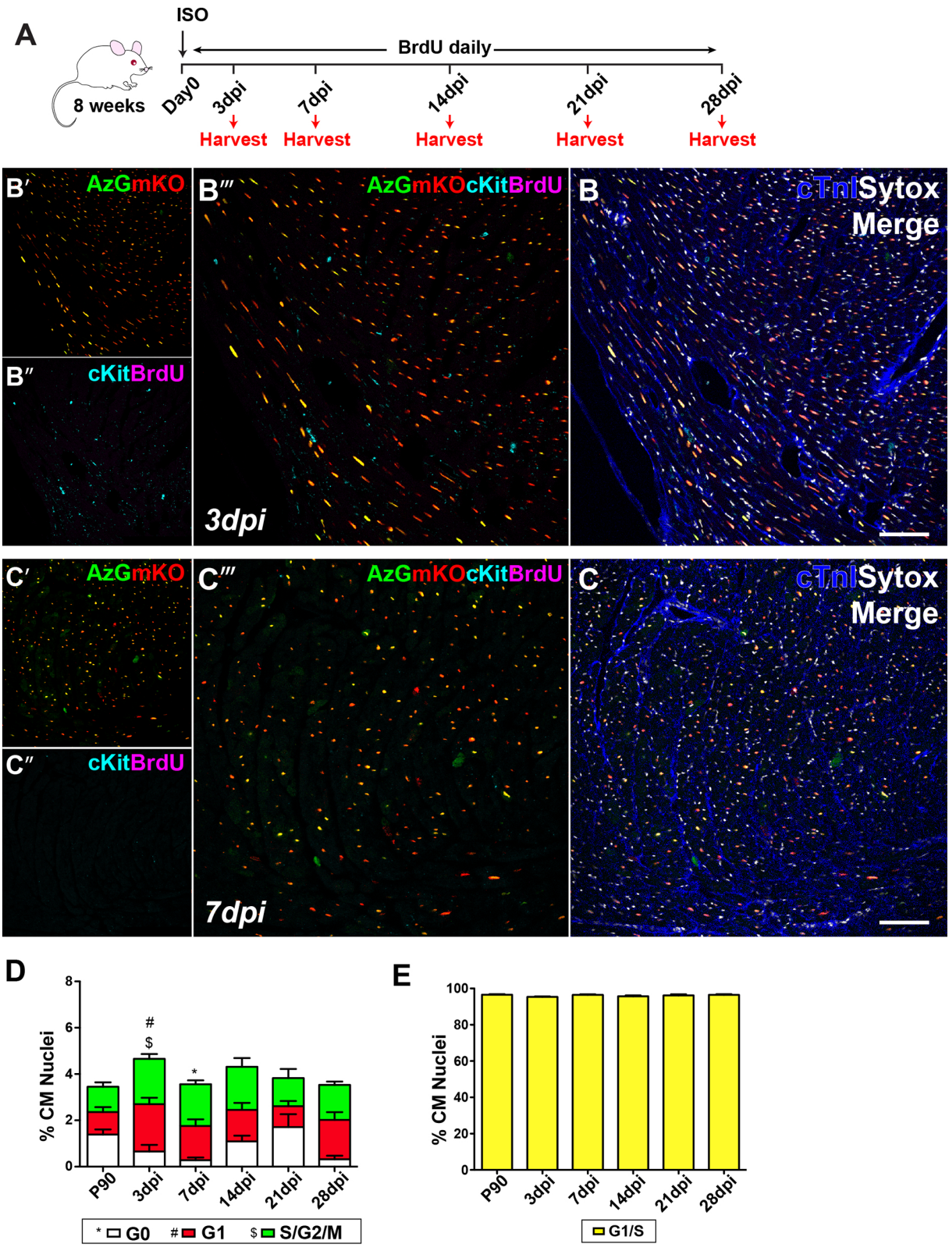

Supplementary Fig S8