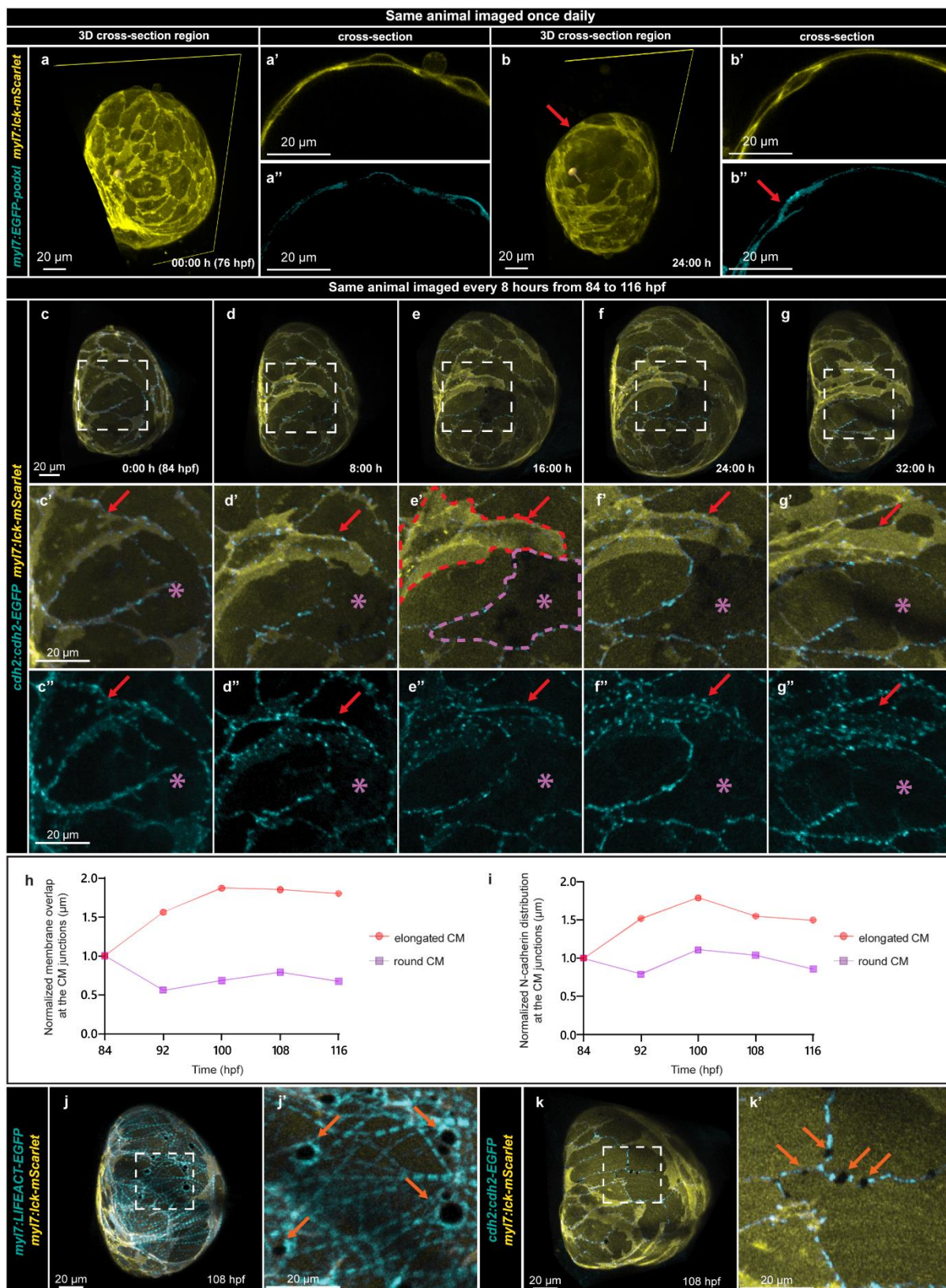
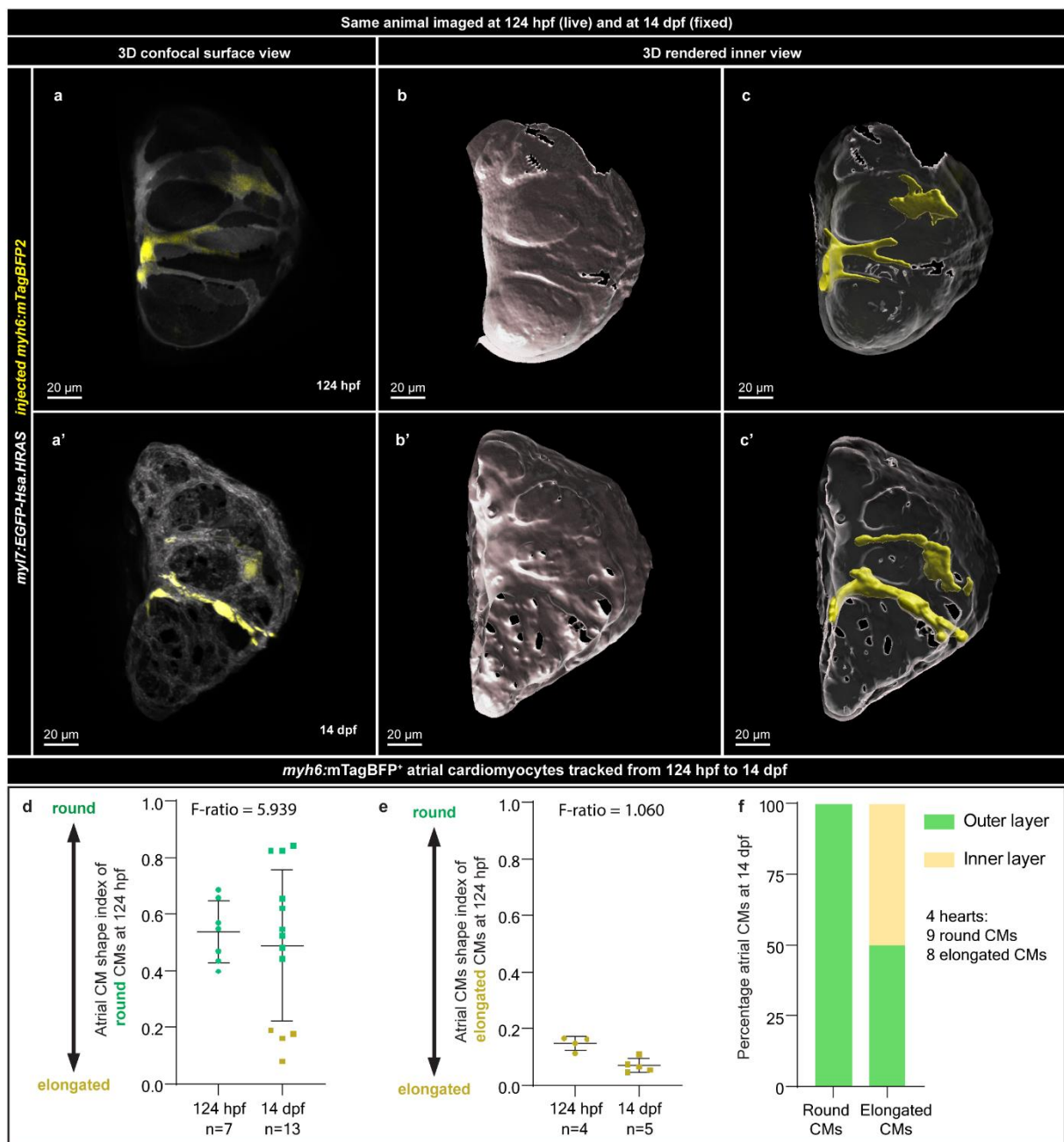
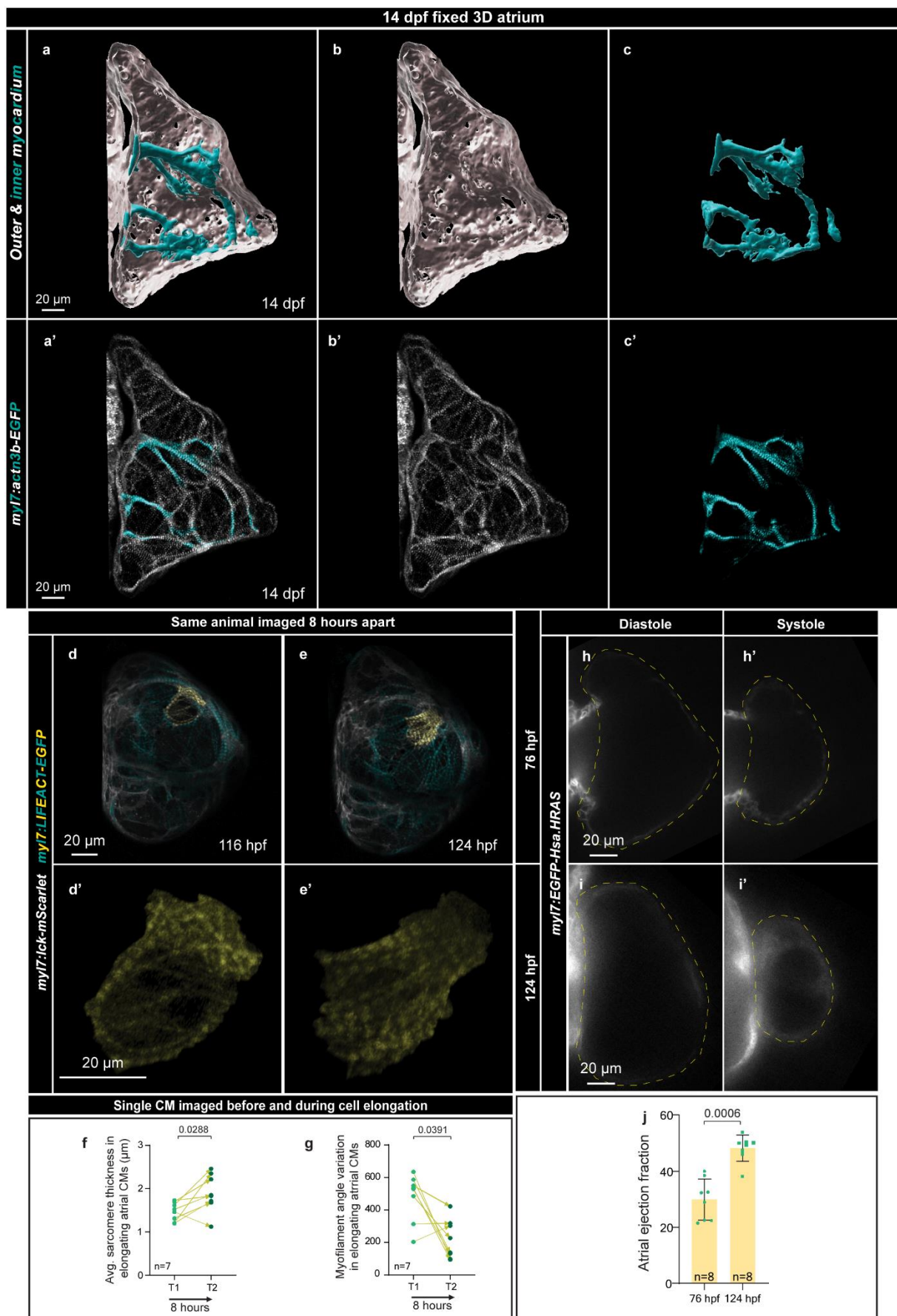




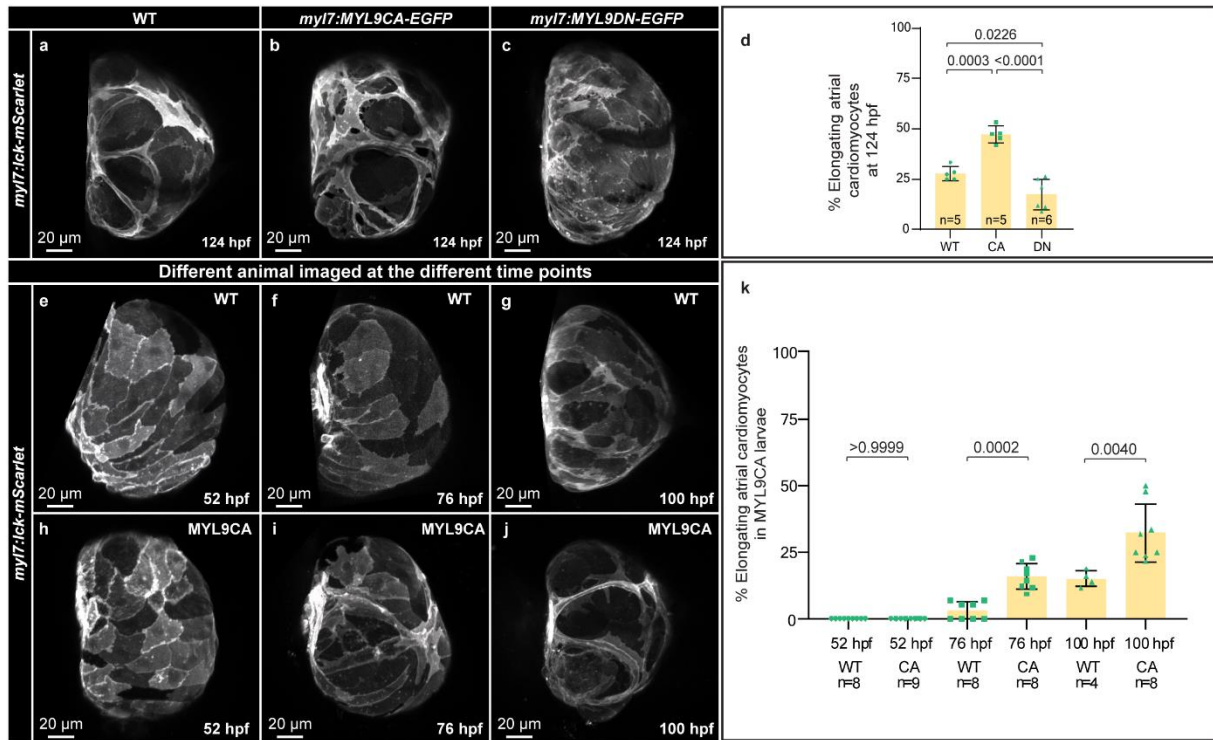
Supplementary Figure 1: Atrial cardiomyocyte intercalation changes apicobasal polarity and N-cadherin distribution. **a-b''**, 3D cross-section regions of confocal images taken from the same atrium at 76 and 100 hpf; CM membranes shown in yellow (*myl7:lck-mScarlet*) and Podocalyxin in cyan (*myl7:EGFP-podxl*); 2D cross-sections (**a'**, **a''**, **b'**, **b''**); red arrows point to elongating atrial CM. **c-g''**, Airyscan images of the same larva imaged every 8 hours from 84 to 116 hpf; CM membranes shown in yellow (*myl7:lck-mScarlet*) and N-cadherin in cyan (*cdh2:cdh2-EGFP*); white squares indicate the zoomed in regions; red arrows point to an elongating CM; purple asterisk marks a round CM; red and purple dashed lines in **c'** outline the analyzed CMs. **h, i**, Changes in the distribution of the mScarlet membrane marker and of N-cadherin in the elongating (red) and round (purple) CMs. **j, j'**, Airyscan images of a 108 hpf atrium with CM membranes in yellow (*myl7:lck-mScarlet*) and CM actin in cyan (*myl7:LIFEACT-EGFP*); white square indicates zoomed in region; arrows point to CM membrane detachments. **k, k'**, Airyscan images of a 108 hpf atrium with CM membranes in yellow (*myl7:lck-mScarlet*) and N-cadherin in cyan (*cdh2:cdh2-EGFP*); white square indicates zoomed in region; arrows point to CM membrane detachments.



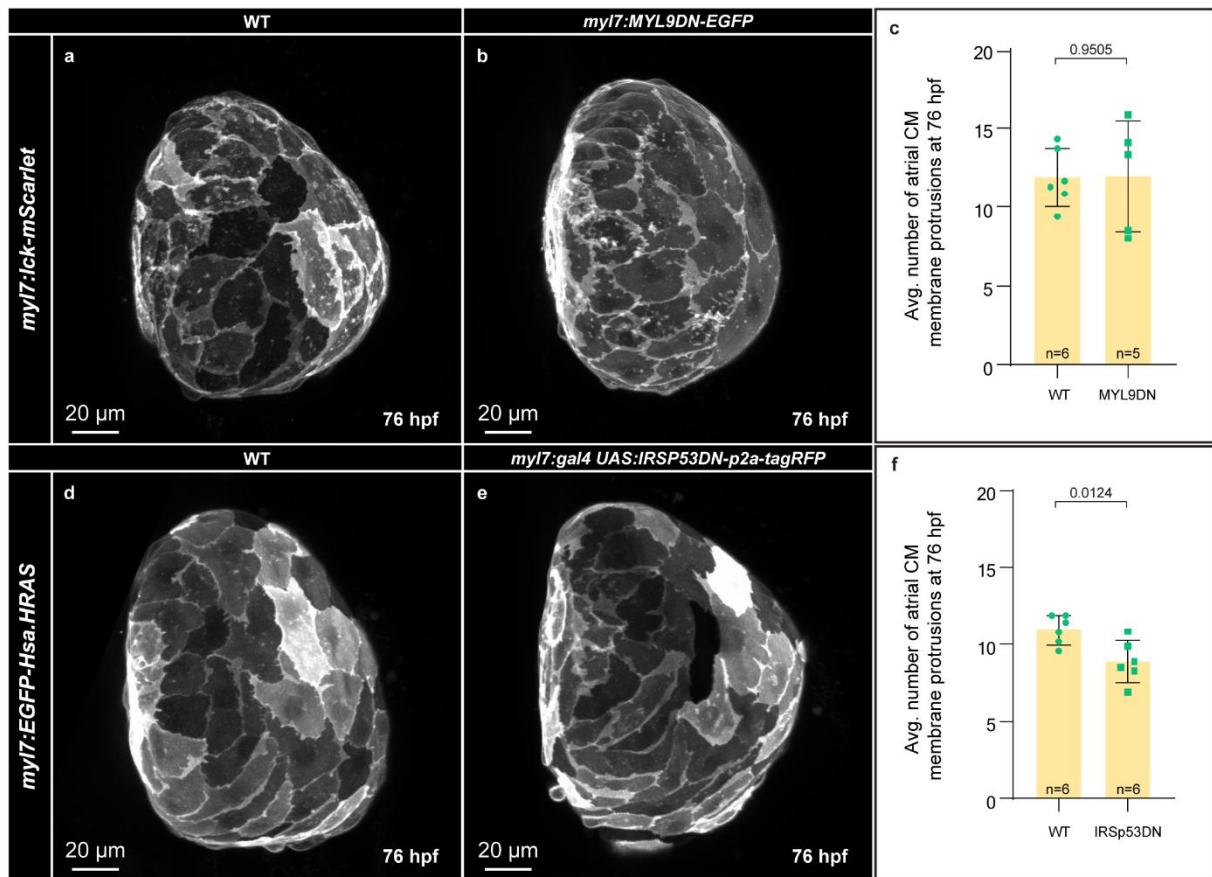
Supplementary Figure 2: Only a subset of atrial cardiomyocytes form the inner muscle structures. **a, a'**, 3D airyscan imaging of the same atrium at 124 hpf (live) and 14 dpf (fixed); CM membranes shown in white (*myl7:EGFP-Hsa.HRAS*) and mosaic CM cytoplasmic marker in yellow (*-0.8myl7:mTagBFP2*). **b, b'**, 3D surface rendering of the membrane marker from **a, a'**. **c, c'**, 3D surface rendering of both the membrane (transparent surface) and cytoplasmic (opaque surface) signals from the images shown in **a** and **a'**. **d, e**, Shape index of *myh6:mTagBFP*⁺ CMs from experiment **a-c'**, determined from the same animals imaged at 124 hpf and 14 dpf (n=7 round CMs at 124 hpf and n=13 CMs at 14 dpf from the 7 round CMs *mTagBFP*⁺ at 124 hpf and their daughter cells; n=4 elongating CMs at 124 hpf and n=5 elongated CMs at 14 dpf from the 7 elongated CMs *mTagBFP*⁺ at 124 hpf and their daughter cells; F-test from two-tailed unpaired Student's t-test, p=0.0389 (**d**), p=0.1485 (**e**)). **f**, Percentage of round or elongating atrial CMs localized in the outer or inner layer of 14 dpf atrium (n=4 hearts, 17 *mTagBFP*⁺ CMs were quantified, 9 round, 8 elongating). Error bars are mean $\pm$ SD.



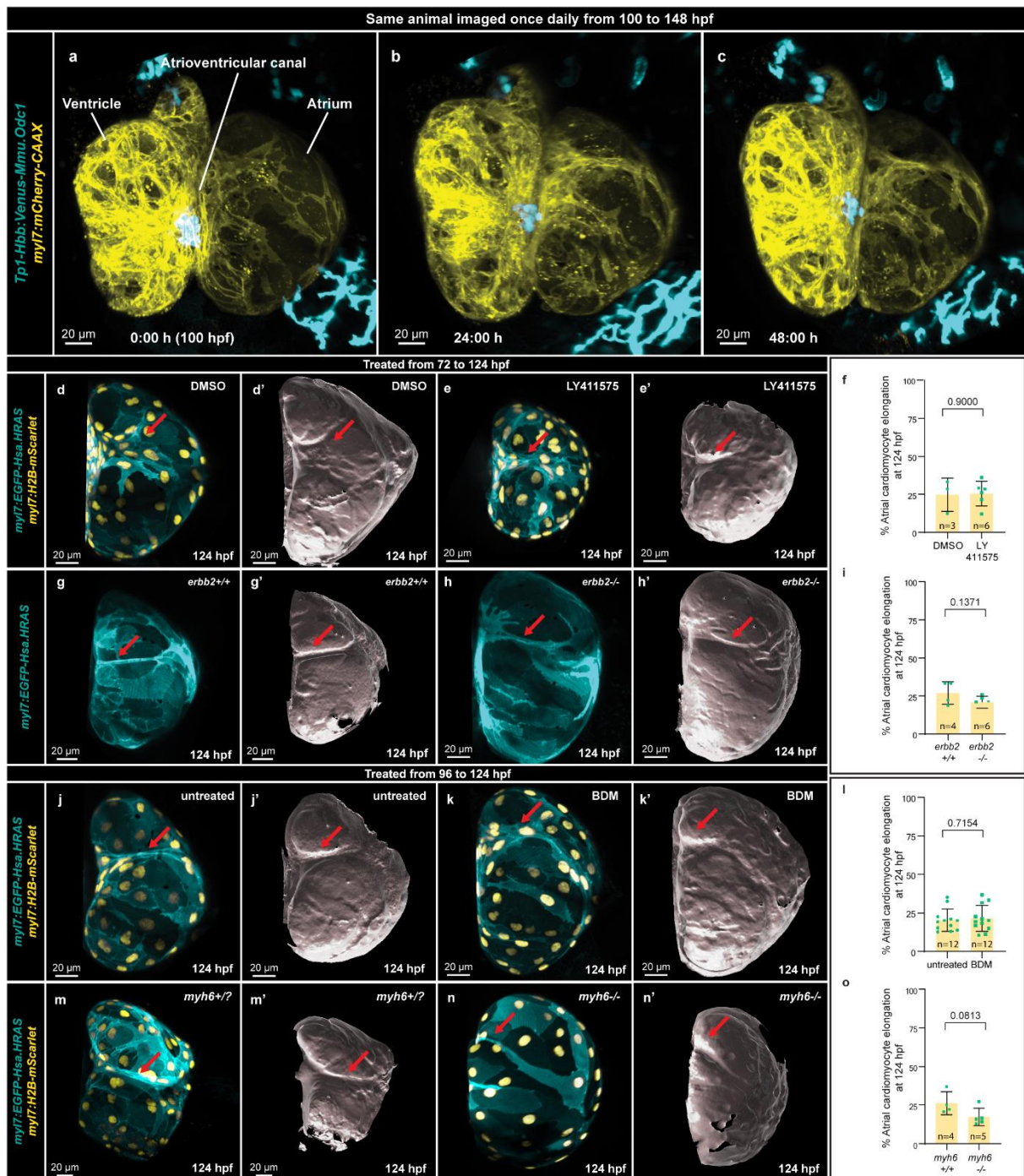
Supplementary Figure 3: Correlation between atrial cardiomyocyte elongation and myofibril maturation. **a-c**, 3D surface rendering of a fixed atrium at 14 dpf; outer layer myocardium shown in white and inner layer myocardium in cyan. **a'-c'**, 3D confocal images of the atrium from which the surface rendering was created; sarcomere Z-bands shown in white for outer layer CMs and in cyan for inner layer CMs (*myl7:actn3b-EGFP*). **d-e'**, 3D confocal images of the same atrium before (116 hpf) and during (124 hpf) CM elongation; CM membranes shown in grey (*myl7:lck-mScarlet*) and CM actin in cyan (*myl7:LIFEACT-EGFP*). Example of myofibrils from an elongating CM tracked over time (yellow). **f, g**, Graphs showing the changes in sarcomere thickness and myofilament angle over time in elongating CMs (n=7; each data point represents the average value in one elongating CM; two tailed paired Student's t-test (f), two-tailed Wilcoxon test (g)). **h-i'**, Images from 2D spinning disk microscopy of a beating atrium at 76 (h, h') and 124 (i, i') hpf (different animals shown); CM membranes shown in grey (*myl7:EGFP-Hsa.HRAS*). **j**, Graph of atrial ejection fraction measured at 76 (prior to CM elongation) and 124 (after CM elongation) hpf (n=8 at 76 hpf and n=8 at 124 hpf; each data point represents one heart; two-tailed Mann-Whitney test) (different zebrafish used). Error bars are mean $\pm$ SD.



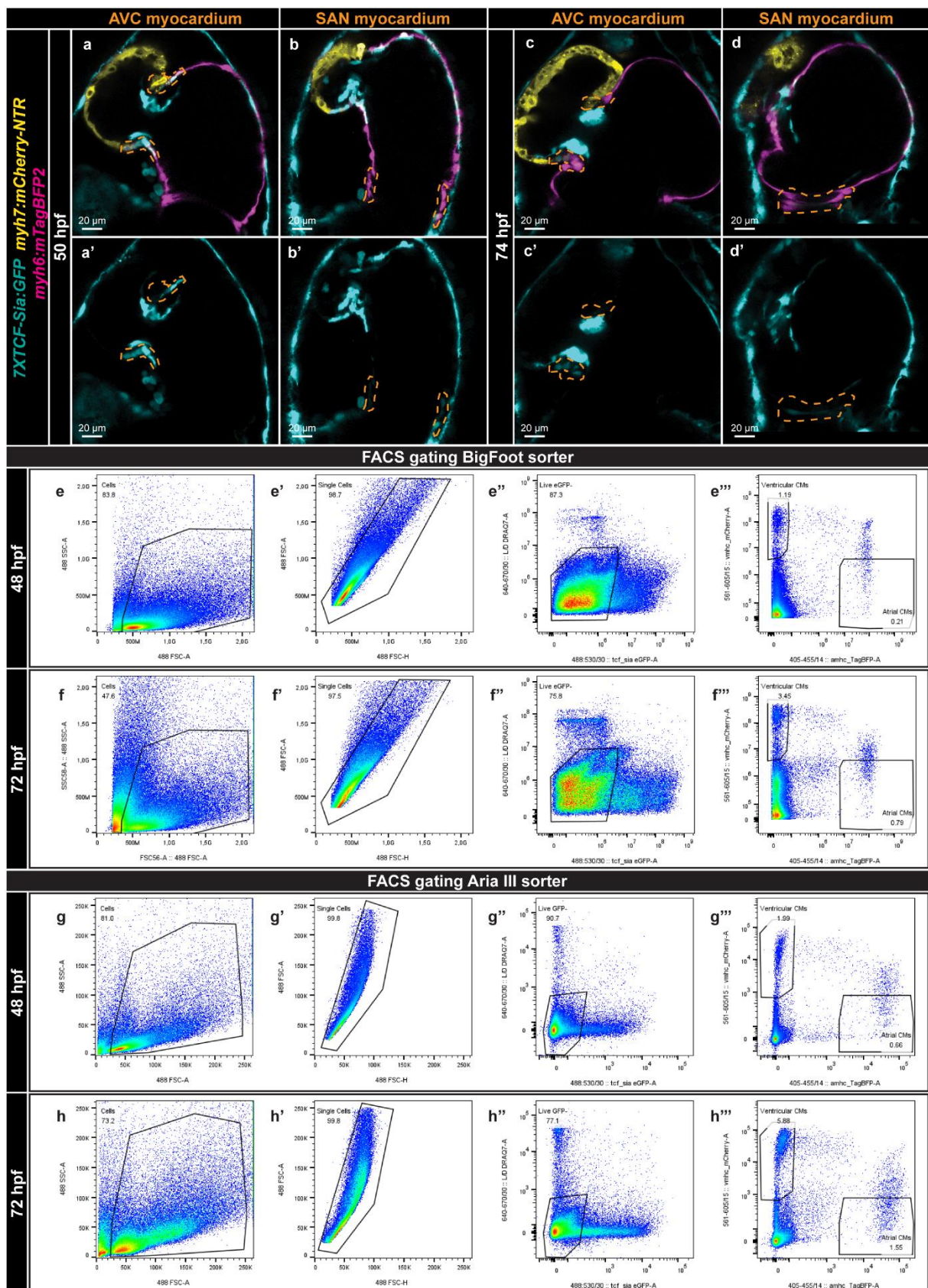
Extended Data Fig. 4: The extent of atrial cardiomyocyte elongation is regulated by cell contractility. **a-c**, 3D airyscan images of atria from 124 hpf wild-type, MYL9^{CA} overexpressing (*myl7:MYL9CA-EGFP*), and MYL9^{DN} overexpressing (*myl7:MYL9DN-EGFP*) larvae. CM membranes shown in white (*myl7:lck-mScarlet*). **d**, Percentage of elongating atrial CMs at 124 hpf in wild-type, MYL9^{CA} and MYL9^{DN} overexpressing hearts (n=5 wild-type and CA and n=6 DN; each data point represents one heart; ordinary one-way ANOVA with Tukey's multiple comparisons test, CA vs DN p-value = 0.23*10⁻⁵). **e-g**, 3D airyscan images of wild-type and MYL9^{CA} overexpressing hearts (*myl7:MYL9CA-EGFP*) atria. The images are from different larvae taken at 52, 76 and 100 hpf. CM membranes shown in white (*myl7:lck-mScarlet*). **k**, Percentage of elongating atrial CMs in 52, 76 and 100 hpf wild-type and MYL9^{CA} hearts (n=8 52 hpf wild-type, n=9 52 hpf CA, n=8 76 hpf wild-type, n=8 76 hpf CA, n=4 100 hpf wild-type, n=8 100 hpf CA; each data point represents one heart; two-tailed Mann-Whitney test for each two conditions compared). Error bars are mean ± SD.



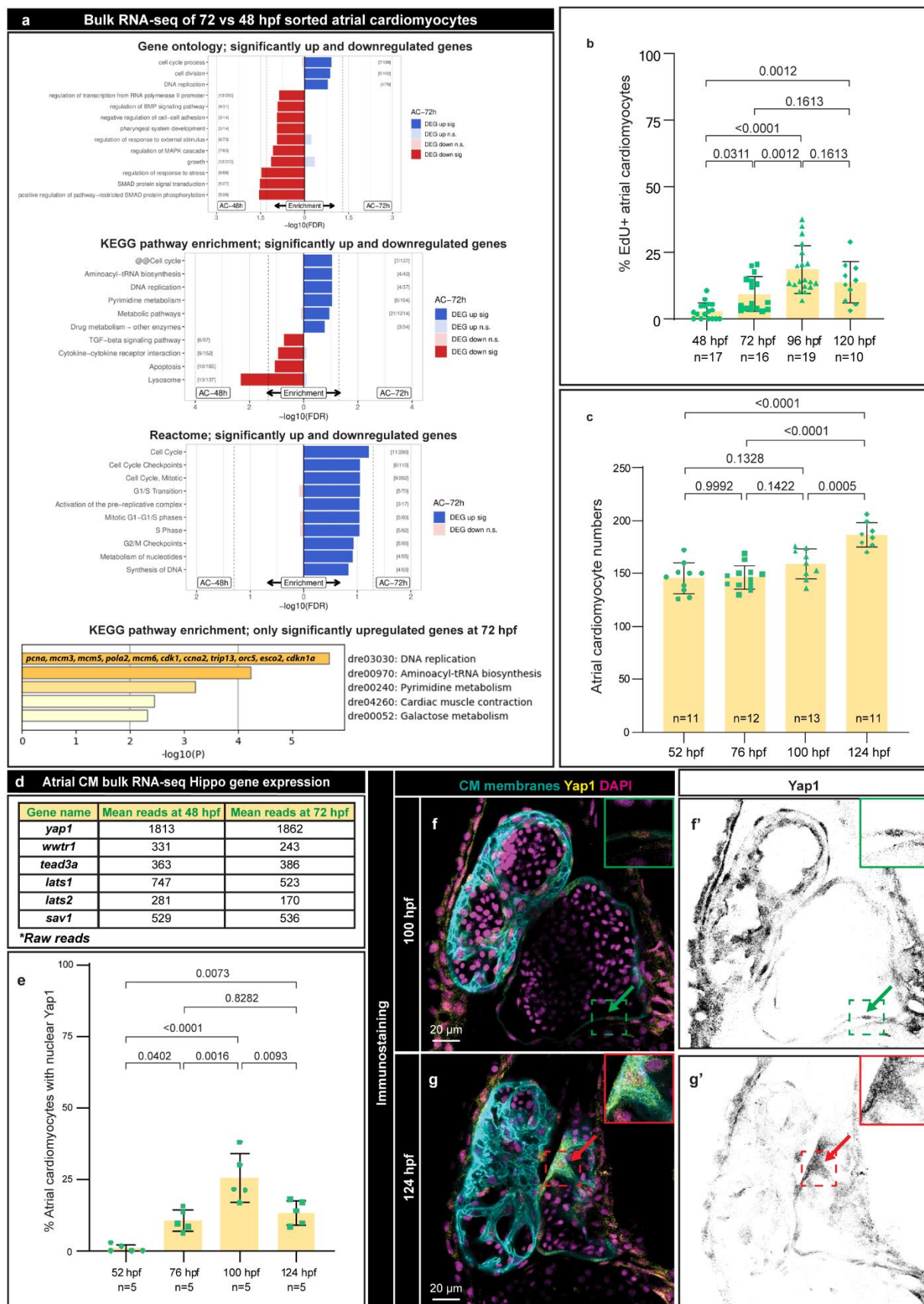
Supplementary Figure 5: Cellular contractility does not appear to affect the number of membrane protrusions in atrial cardiomyocytes. **a, b**, 3D airyscan images of the atrium from 76 hpf wild-type (a) and CM specific MYL9^{DN} overexpressing (b) siblings; CM membranes shown in white (*myl7:lck-mScarlet*). **c**, Number of membrane protrusions per atrial CM and averaged per heart in 76 hpf from wild-type and CM specific MYL9^{DN} overexpressing siblings (n=6 wild-type hearts; n=5 MYL9^{DN} overexpressing hearts; each value represents the average from one heart; two-tailed unpaired Student's t-test). **d, e**, 3D airyscan images of 76 hpf atrium from wild-type (a) and CM specific IRSp53^{DN} overexpressing (b) siblings; CM membranes shown in white (*myl7:EGFP-Hsa.HRAS*). **f**, Number of membrane protrusions per cell and averaged per atrial CM and averaged per heart in 76 hpf from wild-type and CM specific IRSp53^{DN} overexpressing siblings (n=6 wild-type hearts; n=6 IRSp53^{DN} overexpressing hearts; each value represents the average from one heart; two-tailed unpaired Student's t-test). Error bars are mean $\pm$ SD.



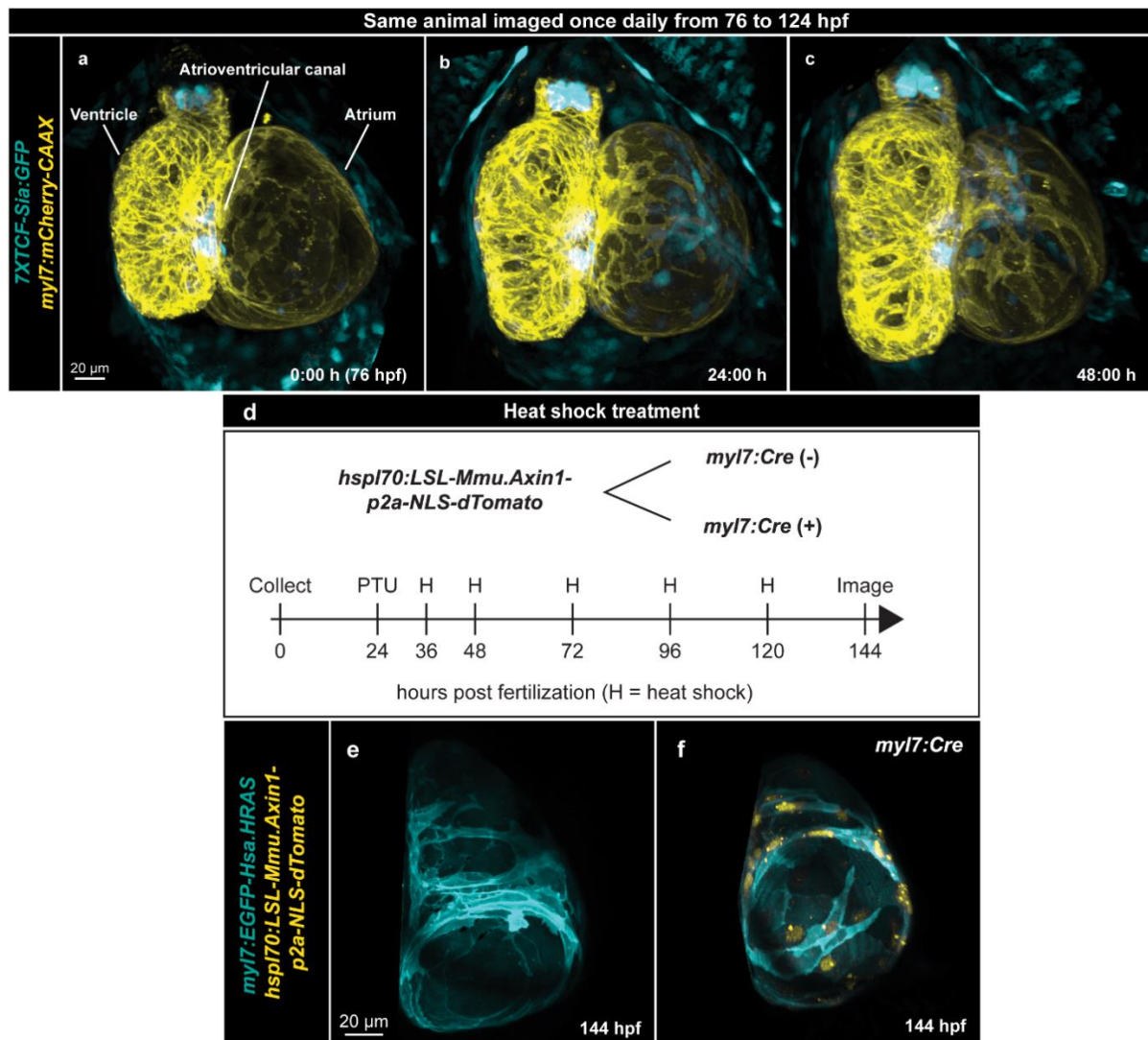
Supplementary Figure 6: Processes required for ventricular trabeculation are not required for atrial morphogenesis. **a-c**, 3D longitudinal confocal images of 100, 124, and 148 hpf hearts; CM membranes shown in yellow (*myl7:mCherry-CAAX*) and Notch reporter expression in cyan (*Tp1-Hbb:Venus-Mmu.Odc1*). **d, e**, 3D airyscan imaging of atria from 124 hpf larvae treated from 72 to 124 hpf with DMSO or LY411575; red arrows point to elongating CMs. **d', e'** Cross-sections through 3D myocardial surface renderings of (d) and (e); arrows point to inner ridges corresponding to the elongating CMs from (d) and (e). **f**, Percentage of elongating atrial CMs from 124 hpf larvae treated with DMSO or LY411575 from 72 to 124 hpf (n=3 DMSO; n=6 LY411575; each data point represents one heart; two-tailed unpaired Student's t-test). **g, h**, 3D airyscan images of atria from 124 hpf homozygous wild-type and *erbb2* mutant sibling larvae; CM membranes shown in cyan (*myl7:EGFP-Hsa.HRAS*); red arrows point to elongating CMs. **g', h'** Cross-sections through 3D myocardial surface renderings of (g) and (h); arrows point to inner ridges corresponding to the elongating CMs from (g) and (h). **i**, Percentage of elongating atrial CMs from 124 hpf homozygous wild-type and *erbb2* mutant siblings (n=4 wild-type; n=6 *erbb2* mutants, each data point represents one heart; two-tailed unpaired Student's t-test). **j, k**, 3D airyscan imaging of atria from 124 hpf larvae treated from 96 to 124 hpf with DMSO or BDM; CM membranes shown in cyan (*myl7:Hsa.HRAS-EGFP*) and CM nuclei in yellow (*myl7:H2B-mScarlet*); red arrows point to elongating CMs. **j', k'** Cross-sections through 3D myocardial surface renderings of (j) and (k); arrows point to inner ridges corresponding to the elongating CMs from (j) and (k). **l**, Percentage of elongating atrial CMs from 124 hpf untreated and BDM treated (n=12 untreated; n=12 BDM; each data point represents one heart; two-tailed unpaired Student's t-test). **m, n**, 3D airyscan imaging of 124 hpf homozygous wild-type and *myh6* mutant siblings; CM membranes shown in cyan (*myl7:EGFP-Hsa.HRAS*) and CM nuclei in yellow (*myl7:H2B-mScarlet*); red arrows point to elongating CMs. **m', n'** Cross-sections through 3D myocardial surface renderings of (m) and (n); arrows point to inner ridges corresponding to elongating CMs from (m) and (n). **o**, Percentage of elongating atrial CMs from 124 hpf homozygous wild-type and *myh6* mutant sibling larvae (n=4 wild-type; n=5 *myh6* mutants; each data point represents one heart; two-tailed unpaired Student's t-test). Error bars are mean $\pm$ SD.



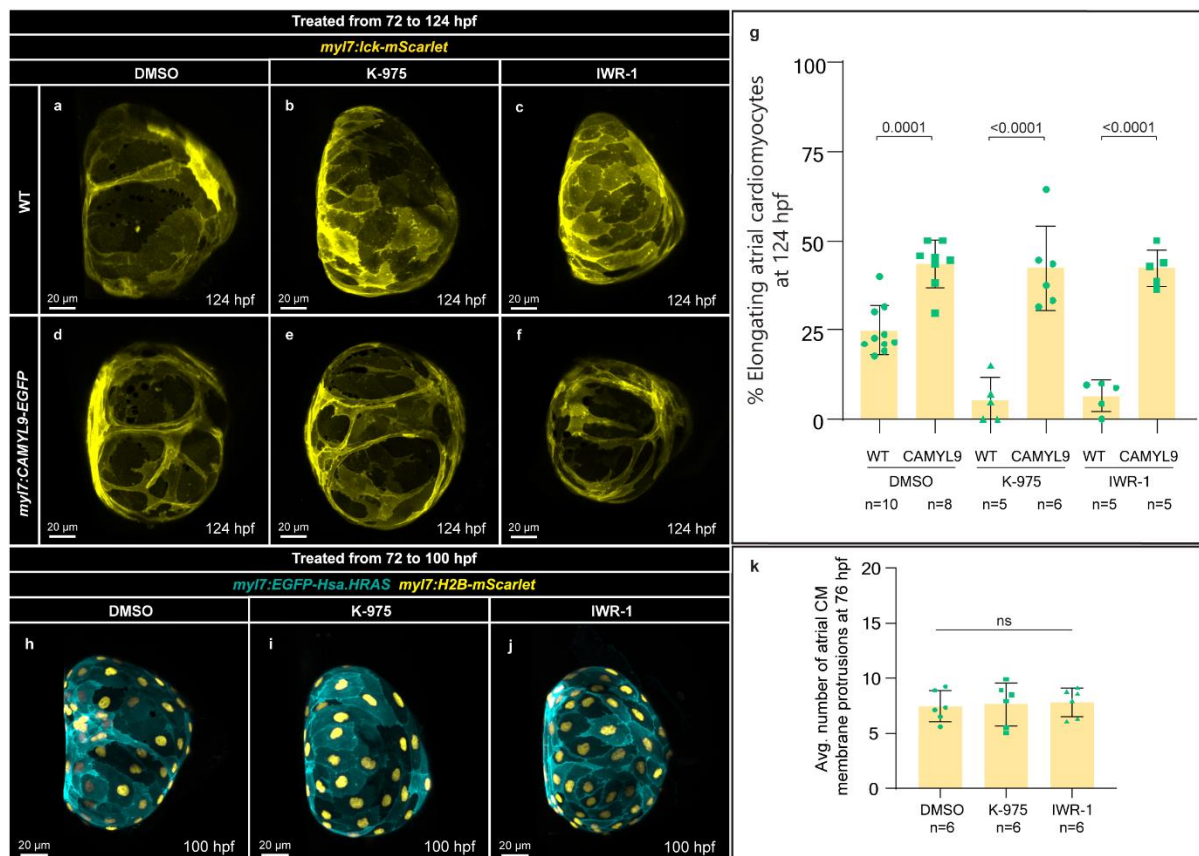
Extended Data Fig. 7: Atrial cardiomyocyte Fluorescence Activated Cell Sorting. **a-d'**, Confocal images of a zebrafish heart at 50 (a-b') and 74 (c-d') hpf; ventricular CMs shown in yellow (*myh7:mCherry-NTR*), atrial CMs in magenta (*-4.5myh6:mTagBFP2*), and AVC and sinoatrial node CMs in cyan (*7XTCF-Sia:eGFP*); AVC and sinoatrial node CMs outlined by the orange dashed lines (a'-d'). **e-f''**, Graphs showing the BigFoot gating strategies to sort atrial CMs at 48 (e-e'') and 72 (f-f'') hpf. **g-h''**, Graphs showing the Aria III gating strategies to sort atrial CMs at 48 (g-g'') and 72 (h-h'') hpf.



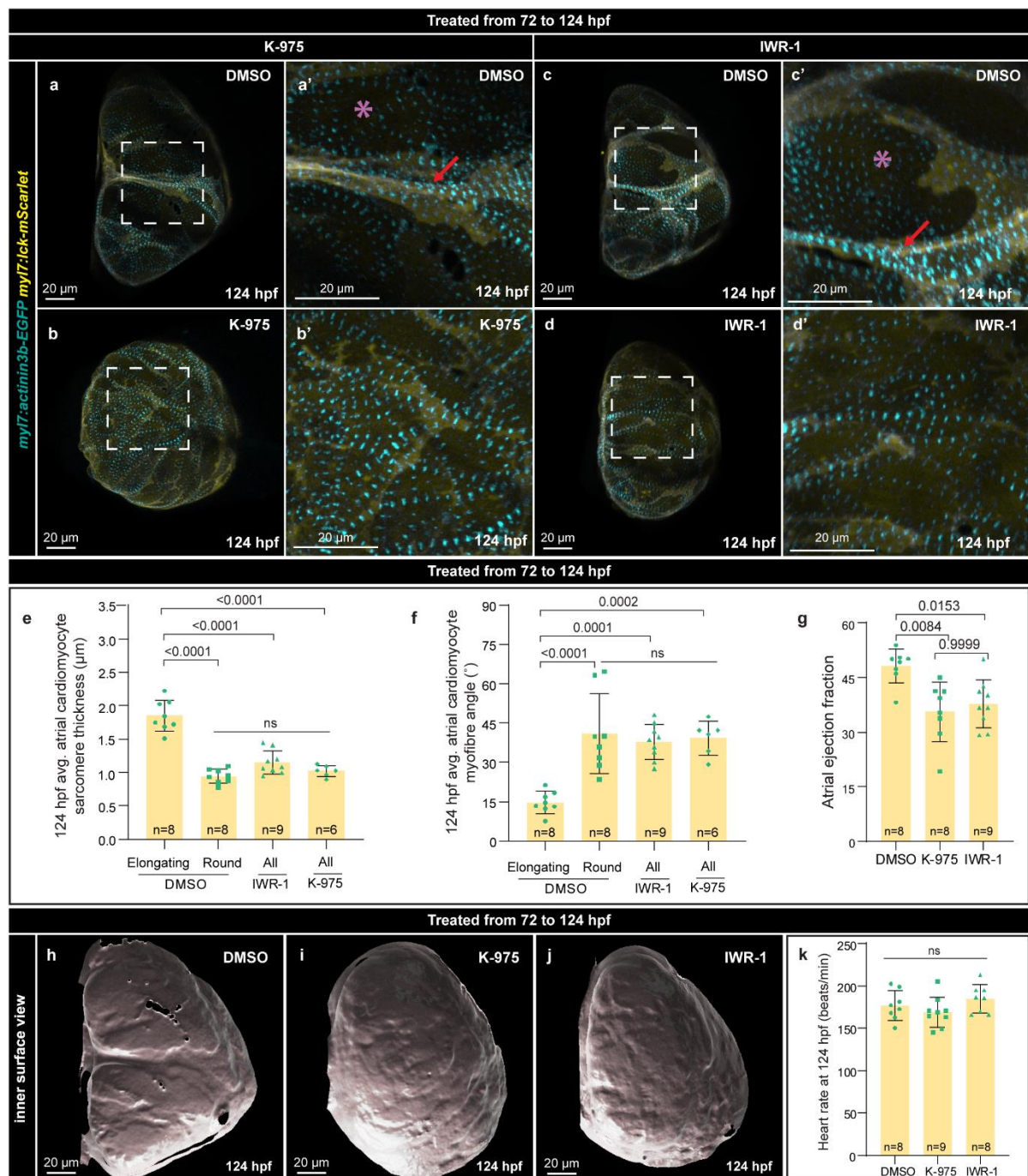
Extended Data Fig. 8: Atrial cardiomyocyte cell division and Hippo pathway gene expression. **a**, Significantly differentially expressed genes in sorted atrial CMs at 72 hpf compared with 48 hpf. **b**, Percentage of EdU⁺ atrial CMs after a 24-hour pulse starting at the indicated times (n=17 48 hpf, n=16 72 hpf, n=19 96 hpf, n=10 120 hpf; each data point represents one heart; ordinary one-way ANOVA with Tukey's multiple comparisons test). **c**, Atrial CM numbers at 52, 76, 100, and 124 hpf (n=11 52 hpf, n=12 76 hpf, n=13 100 hpf, n=11 124 hpf; each data point represents one heart; Kruskal-Wallis test with Dunn's multiple comparisons test). **d**, Hippo pathway gene expression levels from bulk RNA-sequencing data of sorted atrial CMs at 48 and 72 hpf. **e**, Percentage of atrial CMs with nuclear Yap1 at 52, 76, 100, and 124 hpf (n=5 all time points; each data point represents one heart; ordinary one-way ANOVA with Tukey's multiple comparisons test). **f-g'**, Confocal images of hearts immunostained for CM membranes (anti-GFP, cyan) and Yap1 (anti-Yap1, yellow f, g; grey f', g'), and counterstained with DAPI (DNA, magenta) imaged at 100 and 124 hpf; green arrow points to an atrial CM with nuclear Yap1 localization; red arrow points to an elongating atrial CM that does not exhibit nuclear Yap1 localization; squares show the magnified regions. Error bars are mean $\pm$ SD.



Supplementary Figure 9: No TCF-Sia reporter expression in atrial cardiomyocytes, and no significant effect on atrial cardiomyocyte elongation from overexpressing Axin1 in cardiomyocytes. **a-c**, 3D confocal imaging of the same heart taken once daily from 76 to 124 hpf; CM membranes shown in yellow (*myl7:mCherry-CAAX*) and Wnt/ β -catenin reporter expression in cyan (*7xTCF-Sia:GFP*). **d**, Schematic of heat shock treatment (made in Adobe Illustrator by MA). **e, f**, 3D confocal images of atria from larvae that were heat shocked as described in (d); CM membranes shown in cyan (*myl7:EGFP-Hsa.HRAS*) and Axin1 overexpression in yellow (*hspl7:LSL-Mmu.Axin1-p2a-NLS-dTomato*).

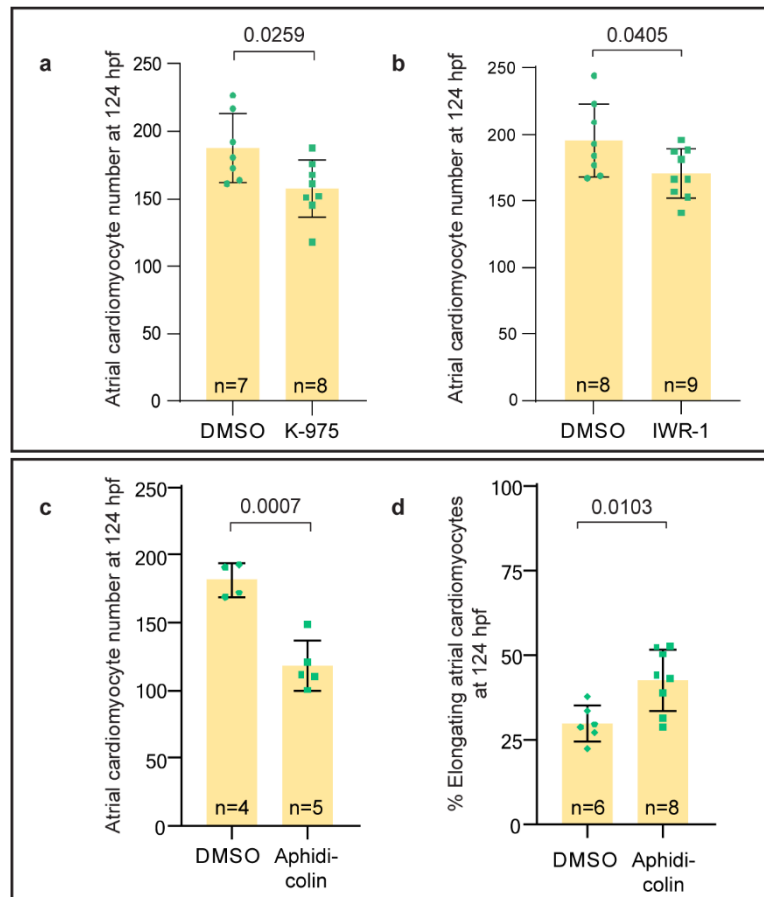


Supplementary Figure 10: Cytoskeletal changes important for atrial cardiomyocyte elongation occur downstream of the Hippo pathway. **a-f**, 3D confocal images of 124 hpf larvae treated from 72 to 124 hpf with DMSO, K-975, or IWR-1; CM membranes shown in yellow (*myl7:lck-mScarlet*); **a-c**, control siblings; **d-f**, CM specific MYL9^{CA} overexpressing larvae (*myl7: MYL9CA-EGFP*). **g**, Percentage of elongating atrial CMs from 124 hpf control siblings and CM specific MYL9^{CA} overexpressing larvae treated from 72 to 124 hpf with DMSO, K-975, or IWR-1 (n=10 DMSO control, n=8, DMSO MYL9^{CA}, n=5 K-975 control, n=6 K-975 MYL9^{CA}, n=5 IWR-1 control, n=5 IWR-1 MYL9^{CA}; each data point represents one heart; ordinary one-way ANOVA with Tukey's multiple comparisons test, K-975 WT vs MYL9^{CA} p-value = 0.28×10^{-8} , IWR-1 WT vs MYL9^{CA} p-value = 0.41×10^{-7}). **h-j**, 3D airyscan images of 100 hpf larvae treated from 72 to 100 hpf with DMSO, K-975, or IWR-1; CM membranes shown in cyan (*myl7:EGFP-Hsa.HRAS*) and CM nuclei in yellow (*myl7:H2B-mScarlet*). **k**, Number of atrial CM membrane protrusions determined per cell and averaged per heart (n=6 all three conditions; ordinary one-way ANOVA with Tukey's multiple comparisons test; DMSO vs K-975 p-value = 0.9780, DMSO vs IWR-1 p-value = 0.9113, K-975 vs IWR-1 p-value = 0.9758). Error bars are mean $\pm$ SD.



Supplementary Figure 11: Loss of atrial cardiomyocyte elongation impairs cardiac

function. **a-c**, 3D confocal images of atria from 124 hpf larvae treated from 72 to 124 hpf with DMSO (**e**, **e'**, **g**, **g'**), K-975 (**f**, **f'**), or IWR-1 (**h**, **h'**); CM membranes shown in yellow (*myl7:lck-mScarlet*) and sarcomere Z-bands in cyan (*myl7:actinin3b-EGFP*). Dashed squares indicate the zoomed in regions; red arrows point to elongating CMs; purple asterisks indicate round CMs. **e**, Atrial CM average sarcomere thickness from 124 hpf larvae treated from 72 to 124 hpf with DMSO, IWR-1, or K-975 (n=8 DMSO; average of 3 round and 3 elongating CMs per heart; n=9 IWR-1, n=6 K-975; average of 6 CMs per heart; each data point represents one heart; ordinary one-way ANOVA with Tukey's multiple comparisons test, DMSO elongating vs round p-value = 0.14×10^{-9} , DMSO elongating vs IWR-1 p-value = 0.17×10^{-7} , DMSO elongating vs K-975 p-value = 0.49×10^{-8} , DMSO round vs IWR-1 p-value = 0.0509, DMSO round vs K-975 p-value = 0.3909, IWR-1 vs K-975 p-value = 0.2941). **f**, Average atrial CM myofibril orientation from 124 hpf larvae treated from 72 to 124 hpf with DMSO, IWR-1, or K-975 (n=8 DMSO; average of 3 round and 3 elongating CMs per heart; n=9 IWR-1, n=6 K-975; average of 6 cells per heart; each data point represents one heart; ordinary one-way ANOVA with Tukey's multiple comparisons test, DMSO elongating vs round p-value = 0.29×10^{-4} , DMSO round vs IWR-1 p-value = 0.9007, DMSO round vs K-975 p-value = 0.988, IWR-1 vs K-975 p-value = 0.991). **g**, Atrial ejection fraction in 124 hpf larvae treated with DMSO, K-975, or IWR-1 (n=8 DMSO and K-975, n=9 IWR-1, each data point represents one heart; Kruskal-Wallis test with Dunn's multiple comparisons test). **h-j**, Cross-sections through 3D reconstructed atrium from 124 hpf larvae treated from 72 to 124 hpf with DMSO, K-975, or IWR-1. **k**, Heart rate in 124 hpf larvae treated from 72 to 124 hpf with DMSO, K-975, or IWR-1 (n=8 DMSO, n=9 K-975, n=8 IWR-1; each data point represents one heart; ordinary one-way ANOVA with Tukey's multiple comparisons test, DMSO vs K-975 p-value = 0.6385, DMSO vs IWR-1 p-value = 0.628, K-975 vs IWR-1 p-value = 0.1715). Error bars are mean $\pm$ SD.



Supplementary Figure 12: Hippo pathway inhibition reduces atrial cardiomyocyte numbers. **a, b,** Atrial CM numbers in 124 hpf larvae treated from 72 to 124 hpf with DMSO, K-975, or IWR-1 (n=7 DMSO, n=8 K-975, n=8 DMSO, n=9 IWR-1; each data point represents one heart; two-tailed unpaired Student's t-test). **c,** Atrial CM numbers in 124 hpf larvae treated from 72 to 124 hpf with DMSO or Aphidicolin (n=4 DMSO, n=5 Aphidicolin; each data point represents one heart; two-tailed unpaired Student's t-test). **d,** Percentage of elongating atrial CMs in 124 hpf larvae treated from 72 to 124 hpf with DMSO or Aphidicolin (n=6 DMSO, n=8 Aphidicolin, each data point represents one heart; two-tailed unpaired Student's t-test). Error bars are mean $\pm$ SD.

Condition	Atrial CM protrusion formation	Atrial CM elongation	Atrial CM orientation
IRSp53 ^{DN} CM overexpression	reduced	reduced	affected
NSC23766 (Rac1 inhibition)	not reduced	reduced	not affected
MYL9 ^{DN} CM overexpression	not reduced	reduced	not analyzed
MYL9 ^{CA} CM overexpression	not analyzed	increased	not analyzed
K-975 (Yap1 inhibition)	not reduced	reduced	affected
IWR-1 (Tankyrase inhibition)	not reduced	reduced	affected

Supplementary Table 1: Relationship between atrial CM protrusions and their elongation.