**Yap Promotes Noncanonical Wnt Signals from Cardiomyocytes for Heart Regeneration**

Shijie Liu, Li Tang, Xiaolei Zhao, Bao Nguyen, Todd R. Heallen, Min Li, Jianxin Wang, Jun Wang, James F. Martin

## MATERIALS AND METHODS

All data and supporting materials have been provided with the published article, and all essential research materials are listed in the Major Resources Table in the Data Supplement.

The sequencing data in this manuscript have been deposited in the Gene Expression Omnibus under accession number GSE179355. All other data are available from the corresponding author upon reasonable request.

### Mice

Mouse studies were performed in accordance with the institutional animal care and use committee at Baylor College of Medicine (Houston, TX). *Myh6-Cas9* mice (C57BL/6 x 129) were a gift from Dr. Olson's lab and have been described previously.[1] *Wls*$^{flox/flox}$ mice (012888, 129S6/SvEvTac), and TCF/LEF:H2B-GFP mice (013752, C57BL/6 x 129) were purchased from The Jackson Laboratory. *Wls* CKO mice were generated by crossing *Wls*$^{flox/flox}$ mice with *Myh6-Cre-Ert2* mice (Jackson Lab, 005657, FVB/N) and were used for generating *Wls* CKO*;TCF/LEF:H2B-GFP* mice. Mixed males and females were used in the study.

All mouse procedures were performed in accordance with institutional and governmental guidelines, and approved by the Baylor College of Medicine Institutional Animal Care and Use Committee.

### Cell Culture of Human CFs

Human CFs were purchased from Lifeline Cell Technology (Frederick, MD). Passage 3-5 cells were cultured with FibroLife S2 Fibroblast Medium (LL-0011, Lifeline Cell Technology) and supplemented with fibroblast growth factor, insulin, ascorbic acid, L-glutamine, hydrocortisone hemisuccinate, 2% fetal bovine serum, gentamicin, and amphotericin B. To inhibit the secretion of Wnt ligands from human CFs, cells were treated with porcupine inhibitor LGK-974 (1 μM, AdooQ Bioscience, LLC, Irvine, CA, A12816) for 48 hours. For treatment with Wnt5a and Wnt9a,

100 ng/ml Wnt5a or Wnt9a was added to cells 24 hours after LGK-974 treatment and incubated for another 24 hours before harvesting the samples.

**Quantitative Real-Time PCR Analysis**

Total RNA was extracted from human CFs by using an RNeasy Micro Kit (Qiagen 74004, Hilden, Germany). RNA concentration was determined with a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, NanoDrop 2000c), and quality was assessed by performing agarose gel electrophoresis. cDNA was synthesized by using an iScript™ cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, 1708890). Quantitative real-time PCR was performed with SYBR Green probes by using the QuantStudio with SYBR Green. Program setting, stage 1, 50 °C 2 min, 95 °C 10 min; stage 2, 95 °C 15 sec, 60 °C 1 min, cycles 40. Results were analyzed as ΔΔCt and expressed as the fold change in transcript levels.

Primers:

| | |
|---|---|
| *POSTN* | 5'-gtctttgagacgctggaagg-3' |
| | 5'-agatccgtgaaggtggtttg-3' |
| *FN1* | 5'-accaacctacggatgactcg-3' |
| | 5'-gctcatcatctggccatttt-3' |
| *COL1A1* | 5'-gtgctaaaggtgccaatggt-3' |
| | 5'-ctcctcgctttccttcctct-3' |
| *COL1A2* | 5'-ctgcaagaacagcattgcat-3' |
| | 5'-ggcgtgatggcttatttgtt-3' |
| *COL3A1* | 5'-tacggcaatcctgaacttcc-3' |
| | 5'-gtgtgtttcgtgcaaccatc-3' |
| *COL15A1* | 5'-gttgtccacctaccgagcat-3' |
| | 5'-agccatgccaaatgactttc-3' |
| *ACTB* | 5'-ggacttcgagcaagagatgg-3' |
| | 5'-agcactgtgttggcgtacag-3' |

**AAV Production and Delivery**

Two gRNAs against exon 2 of mouse *Wls* were designed by using the Benchling website (https://benchling.com). gRNA activity was first tested in P19 cells by using the PX-458 backbone, which contains a gRNA scaffold and SpCas9 (Addgene, Watertown, MA, #48183). A surveyor assay was used for testing the activity of gRNA according to the manufacturer's instructions (IDT 706020). The first gRNA was cloned into the 1179_pAAV-U6-BbsI-gRNA-CB-EmGFP backbone (Addgene #89060) by using BbsI cloning sites. The second gRNA, including the U6 promoter and gRNA scaffold, was inserted behind the first gRNA scaffold by using XbaI and HindIII cloning sites to generate a plasmid with two gRNAs for *Wls*. The plasmid was then submitted to the IDDRC Neuroconnectivity Core at Baylor College of Medicine (Houston, TX) for virus packaging. *CM-Cas9* mice were subcutaneously injected with $1\times10^{11}$ viral particles at P3, and LAD-O was performed at P4. Mice were then subjected to echocardiography at 4, 6, and 8 weeks after LAD-O. The Wls gRNA sequences were as follows: Wls-gRNA1: ACAAGATGGCTGGCGCCTTG; Wls-gRNA2: ACACATTTTATTGCCGTGTA.

**LAD-O**

To generate *Wls* CKO mice, TAM was subcutaneously injected daily from P1-P3 at a dose of 40mg/kg to induce Cre/loxp recombination. LAD-O was performed on P2 mice as previously described.[2] Control and mutant mice were randomly treated with LAD-O. Briefly, Nylon sutures (8-0 nonabsorbable) were used to occlude the LAD. Proper occlusion was marked by blanching of the myocardium seen upon visual inspection during tissue collection. Vicryl sutures (6-0 absorbable) were used to close the thoracic cavity. The entire procedure required approximately 12 minutes from the onset of hypothermia to recovery. Sham procedures excluded placement of a suture around the LAD. Mice were subjected to echocardiography 4 weeks after occlusion. After mice were euthanized, hearts were dissected out and processed for histology and immunocytochemistry. Fibrotic scar size measurements were obtained by using automated image segmentation in MIQuant, an open source code for Matlab.[3]

**Echocardiography**

Cardiac function was determined by using echocardiography (FUJIFILM VisualSonics Inc., Toronto, Canada, Vevo 2100, 40 MHz, 550S probe). After alignment in the transverse B-mode with the papillary muscles, cardiac function was measured on M-mode images. At lease 5 mice were used for each group. Group sizes for in vivo studies vary, as indicated in the figure legend. Sample sizes were adequately powered to observe the effects based on previous reports[4]. The Echo results were analyzed blindly.

**Protein Analysis**

For Western blot analysis, proteins were extracted from tissues as previously described.[5] Briefly, CM and non-CMs from mouse hearts were homogenized and lysed in 0.5% NP-40 lysis buffer (50 mM Tris-HCL, 150 mM NaCl, 0.5% NP40 addition with cOmplete™, EDTA-free Protease Inhibitor, and PhosSTOP™ from Roche Holding AG, Basel, Switzerland). 4–20% Mini-PROTEAN® TGX™ Precast Protein Gels (Bio-rad, 4561094) were used to resolve the proteins. Protein bands were detected by using either Pico (Thermos Fisher Scientific) or Dura (SuperSignal™ West Dura Substrate) enhanced chemiluminescence systems. Antibodies used were as follows: rabbit anti-Wls (Abcam, Cambridge, United Kingdom, ab82897), rabbit anti-ROR1 (Cell Signaling Technology, Danvers, MA, #4102), rabbit anti-LRP6 (Cell Signaling, #3395), rabbit anti β-catenin (Cell Signaling, #9562), rabbit anti-Axin2 (Abcam, ab32197), and rabbit anti-GAPDH (Abcam, ab9485). Secondary antibodies: Goat Anti-Rabbit IgG (H + L)-HRP Conjugate (Bio-rad, 1706515), goat anti-Mouse IgG-HRP (Santa cruz, Dallas, TX, sc-2055).

**Histology and Immunofluorescence**

Hearts were harvested and fixed in 10% formalin at 4°C overnight and then processed as paraffin or frozen sections for downstream applications. For paraffin sections, hearts were dehydrated in

alcohol gradients after fixation, treated with xylene, and embedded in paraffin. Slides were sectioned at 7-μm intervals for trichrome staining and immunofluorescence staining, Citrate Buffer, pH 6.0 was used for the antigen retrieval, sections were permeabilized with 0.2% Triton X-100 in PBS, then blocking with 10% Donkey serum (Sigma, D9663). Primary antibodies were diluted in 10% Donkey serum and incubated with sections for overnight. After incubated with Biotinylated secondary antibodies, sections were incubated with fluorophores conjugated Streptavidin for protein detection, fluorophores conjugated Wheat Germ Agglutinin were used to stain the cell boundary. For frozen sections, hearts were dehydrated in 15% and 30% sucrose gradients after fixation and then embedded into optimal cutting temperature compound (V.W.R. Cat#25608-930) for sectioning. Slides were sectioned at 10-μm intervals for immunofluorescence staining, sections were permeabilized with 0.2% Triton X-100 in PBS, then blocking with 10% Donkey serum, and the following procedures were similar to the paraffin section. Antibodies used were as follows: chicken anti-GFP (Abcam ab13970), rabbit anti-Wls (Abcam ab82897), mouse anti-troponin T (Thermo Fisher Scientific #MS-295), rat anti-pHH3 (Abcam ab10543), rabbit anti-β-catenin (Cell Signaling #9562), rabbit anti-vimentin (Abcam, ab92547), rabbit anti-collagen I (Abcam ab21286). Horse Anti-Rabbit IgG Antibody (H+L), Biotinylated (Vector Lab, Burlingame, CA, BA-1100), Goat Anti-Chicken IgY Antibody (H+L), Biotinylated (Vector Lab, BA-9010), Goat Anti-Rat IgG Antibody (H+L), Biotinylated (Vector Lab, BA-9400), Horse Anti-Mouse IgG Antibody (H+L), Biotinylated (Vector Lab, BA-2000), Streptavidin, Alexa Fluor™ 488 conjugate (Thermo Fisher Scientific, S32354), Streptavidin, Alexa Fluor™ 546 conjugate (Thermo Fisher Scientific, S11225), Wheat Germ Agglutinin, Alexa Fluor™ 594 conjugate (Thermo Fisher Scientific, W11262). Mouse samples were randomly selected for analyses. Image analysis was performed in a blinded or unblinded fashion. To distinguish genuine target staining, secondary antibody only controls were used. Please see the Major Resources Table in the Supplemental Materials.

The Masson's trichrome staining were used paraffin sections and followed the protocol in Abcam website: https://www.abcam.com/ps/products/150/ab150686/documents/Trichrome-Stain-Kit-protocol-book-v3d-ab150686%20(website).pdf

**EdU Incorporation Assay**

One week after mice underwent LAD-O at P2, mice were injected subcutaneously with 0.15 mg of EdU 4 hours before euthanization. Hearts were dissected, fixed with 10% neutral buffered formalin, and processed for paraffin embedding. Tissues were sectioned at 7-μm intervals. EdU incorporation was detected by using the Click-it EdU Imaging Kit (Life Technologies Corporation, Carlsbad, CA). Imaging of tissue slides was performed with a Leica TCS SP5 confocal microscope, and images were processed by using Leica LAS AF software (Leica Microsystems GmbH, Wetzlar, Germany).

**CM Isolation**

The isolation of adult CMs was performed as previously described.[6] Briefly, ~2 weeks old hearts were dissected from the mice and perfused in retrograde with HEPES-Tyrode's buffer (130 mM NaCl, 5.4 mM KCl, 0.5 mM MgCl, 0.33 mM $NaH_2PO_4$, 0.25 mM HEPES, 22 mM glucose; 37°C; pH 7.4) containing 100 mg/ml collagenase (collagenase type 2, Worthington) using the Langendorff method. Hearts were then mechanically sheared and filtered through a 100-mm mesh filter. The single-cell suspension was centrifuged at 20g for 3 minutes. The pellets, which contained the majority of CMs, were washed twice with HEPES-Tyrode's buffer containing $CaCl_2$ and were collected after centrifugation at 20g. For the collection of non-CMs, the supernatant was removed and centrifuged at 1000 rpm for 5 minutes. For P16 mouse hearts, the concentration of collagenase was reduced to 40 mg/ml.

**RNA-seq**

Total mRNA was extracted from isolated CMs and non-CMs by using the RNAeasy Mini Kit (Qiagen). RNA quality and concentration were determined by using a fragment analyzer (Advanced Analytical Technologies, Inc., Santa Clara, CA). RNA-seq libraries were constructed by using the Stranded RNA-seq Kit with Ribo Erase (Kapa Biosystems Inc., Wilmington, MA) with custom Y-shaped adapters. Paired-end 2x75 bp sequencing was performed for RNA-seq libraries with an Illumina Nextseq instrument (DNA Link). Reads were mapped to the mouse genome (mm10) by using STAR[7] with the parameters of "--runMode alignReads --outFilterScoreMinOverLread 0 --outFilterMatchNminOverLread 0 --outFilterMismatchNmax 2 --quantMode GeneCounts –outSAMty". The raw read counts were used as input of DESeq2,[8] in which the reads were normalized by the internal library size correction model. Then, differential expression analysis was performed with the default setting. GO analysis was performed using Metascape [http://metascape.org] and was displayed with GO plot.

**Yap ChIP-seq Data Incorporation**

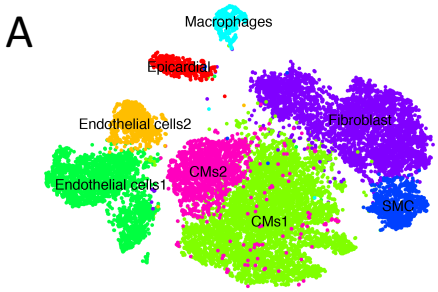
Yap ChIP-seq data was used from GSM2220157[9] and mapped to the mouse genome (mm10) with LiftOver (https://genome.ucsc.edu/cgi-bin/hgLiftOver). Control and Yap5SA ATAC-seq reads, centered at Yap ChIP-seq peaks, were plotted as heat maps and profile plots by using deepTools3 (plotProfile). Motif discovery analysis was performed with HOMER (findMotifsGenome.pl). Yap ChIP-seq targets in our RNA-seq data set were determined by taking Yap ChIP-seq peaks that contained open Tead motifs (ATAC-seq average normalized read depth >2 over the peak length).

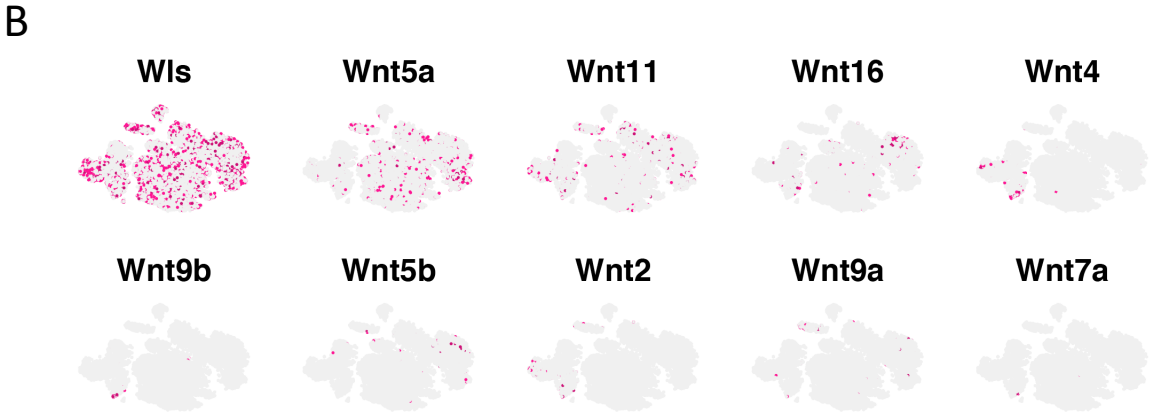
**Statistics**

Data are presented as the mean ± standard error of the mean. Quantitative data for two groups were statistically evaluated using the Mann-Whitney U-test or the two-tailed paired t test. Normality was tested by quantile-quantile plot and Shapiro-Wilk test and equality of variance was

tested using the Brown-Forsythe test or Bartlett's test. For comparisons among multiple groups, we used one-way analysis of variance with Tukey's pairwise post-hoc test. A p-value of less than 0.05 was considered significant for all analyses ($P<0.05$). p-value were showed in each statistical figure.

## SUPPLEMENTARY FIGURES





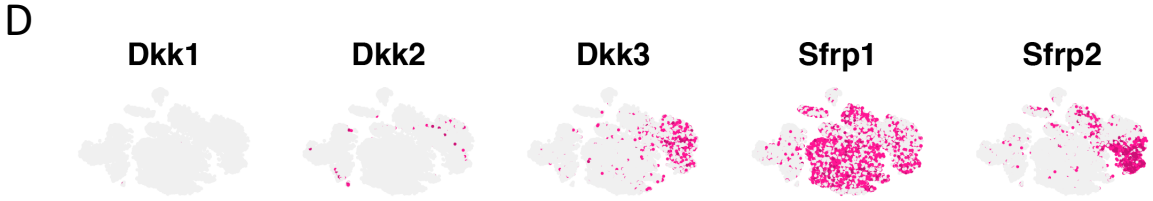


**Figure I. Expression of Wnt components in the E14.5 heart.**

**A**, Classification of cell clusters in the E14.5 mouse heart. **B**, Expression pattern of *Wls* and select Wnt ligands in the E14.5 heart. **C-D**, Expression pattern of Wnt receptors **(C)** and Wnt inhibitors **(D)**.

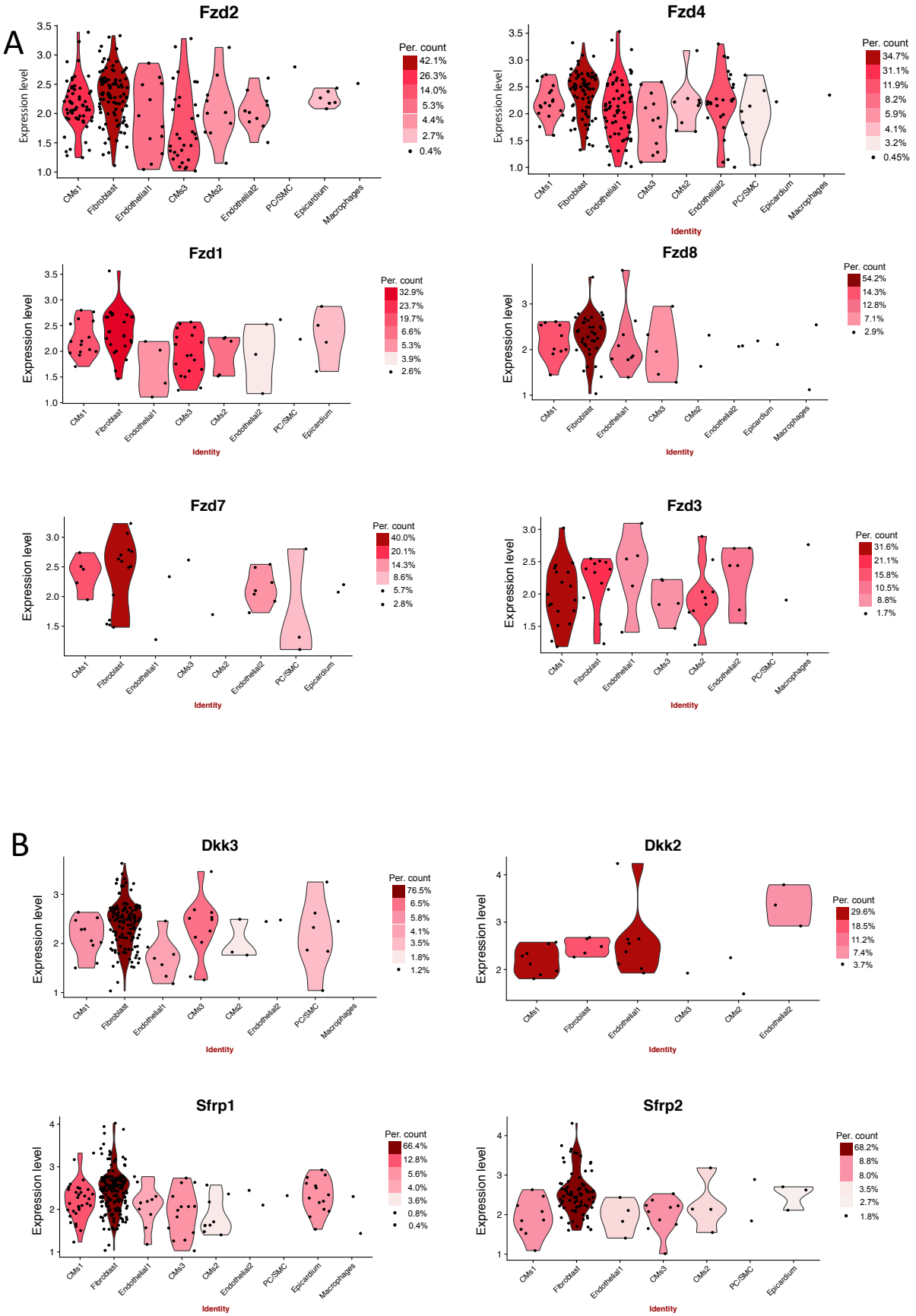

A
Fzd2
Fzd4
Fzd1
Fzd8
Fzd7
Fzd3
B
Dkk3
Dkk2
Sfrp1
Sfrp2
Expression level
Identity
Per. count
CMs1
Fibroblast
Endothelial1
CMs3
CMs2
Endothelial2
PC/SMC
Epicardium
Macrophages

**Figure II. Quantified expression of Wnt components in the P6 heart.**

**A, B**, The expression of Wnt receptors **(A)** and inhibitors **(B)** in different cell types. Dots represent cells with gene expression as indicated. The position of the dot related to the Y-axis represents the expression level. Color intensity represents the percentage of cell distribution. Most of *Fzd2*- and *Fzd4*-expressing cells are fibroblasts; the expression levels of these genes are higher in fibroblasts than in other cell types.

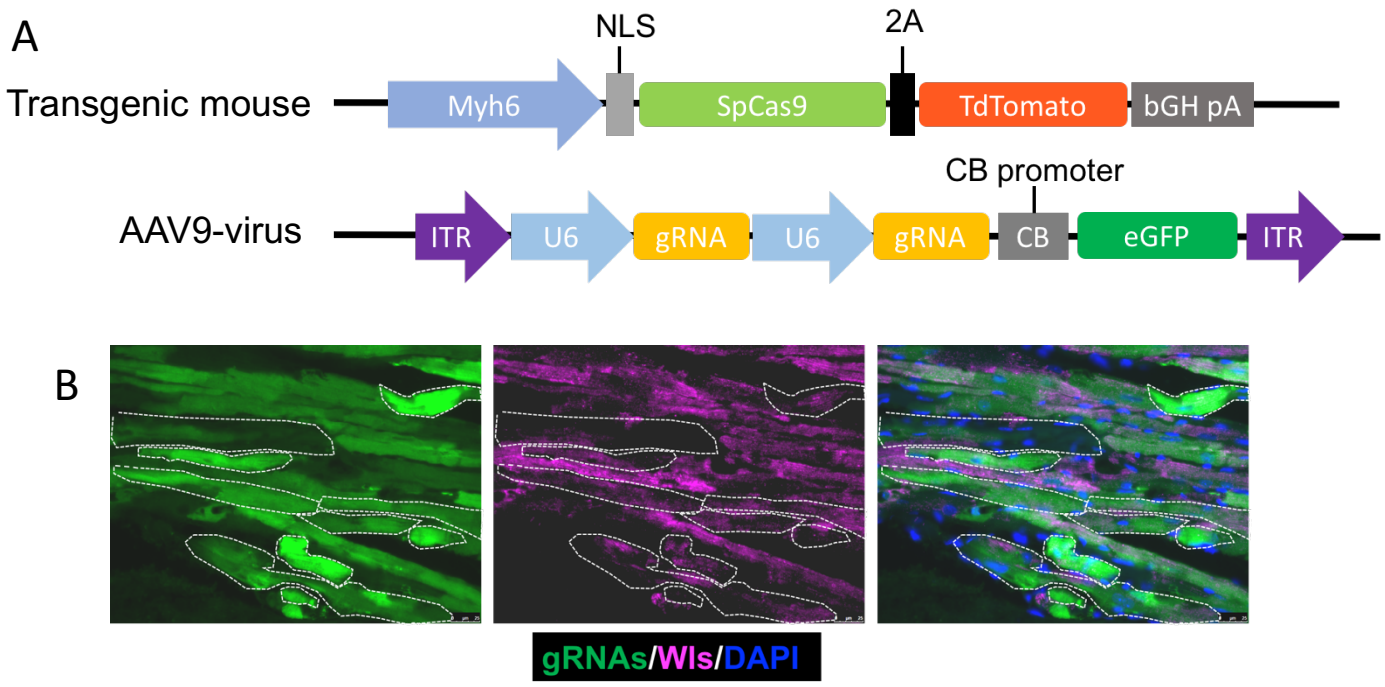
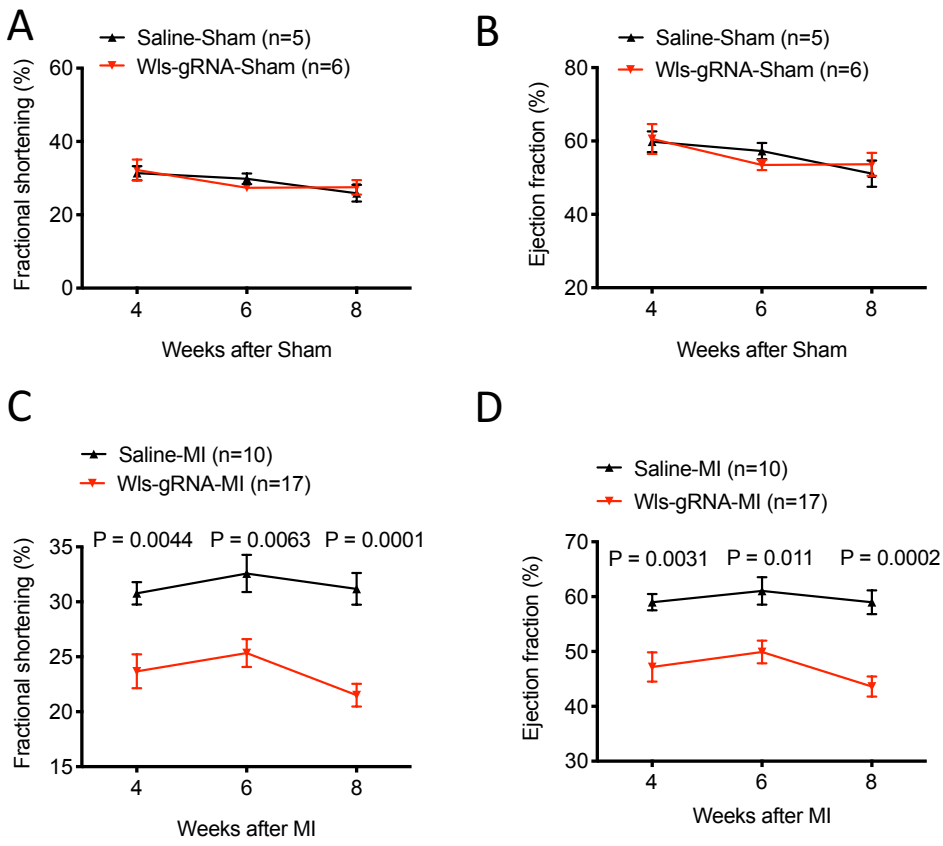
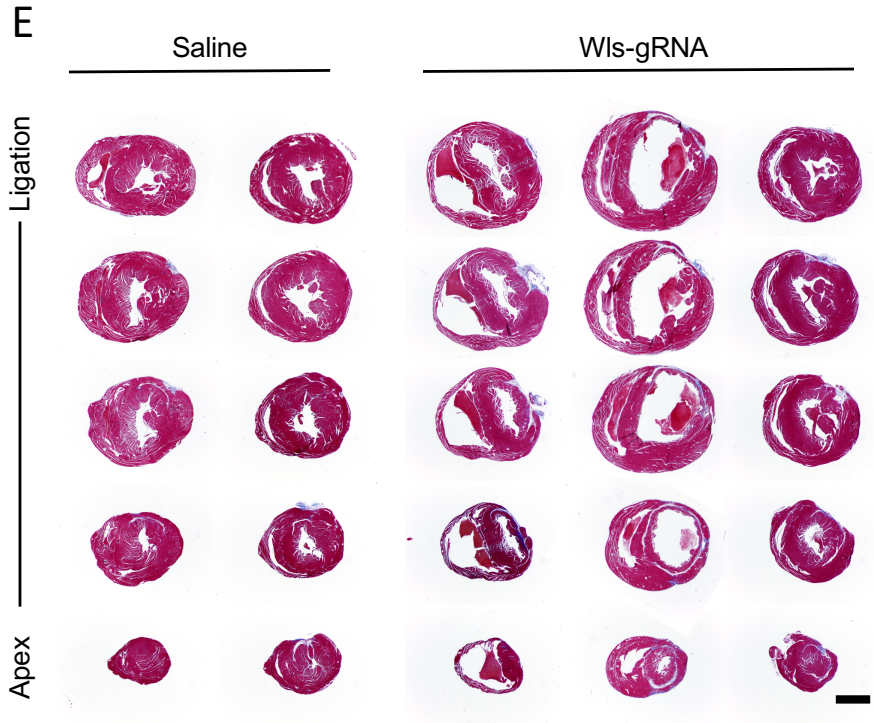

A
Transgenic mouse
Myh6
NLS
SpCas9
2A
TdTomato
bGH pA
AAV9-virus
ITR
U6
gRNA
U6
gRNA
CB promoter
CB
eGFP
ITR
B
gRNAs/Wls/DAPI
C
Fractional shortening (%)
P=0.246
40
30
20
10
0
Saline
Wls-gRNA
D
Ejection fraction (%)
P=0.329
80
60
40
20
0
Saline
Wls-gRNA
E
Control
Cas9-Wls

**Figure III. Wls is dispensable for heart function in homeostasis.**

**A**, Schematic of constructs used to generate *CM-Cas9* and *AAV9-Wls*-gRNA transgenic mice. **B**, GFP and Wls staining in a mouse heart injected with *AAV9-Wls*-gRNA. The dashed line outlines the CMs infected with *Wls-KD*. **C, D**, Heart function assessed by fractional shortening and ejection fraction. Saline indicates saline-injected WT mice. Mann–Whitney test was used for the comparison. **E**, Trichrome staining. Scale bars=1 mm.


A
Saline-Sham (n=5)
Wls-gRNA-Sham (n=6)
Fractional shortening (%)
Weeks after Sham
B
Saline-Sham (n=5)
Wls-gRNA-Sham (n=6)
Ejection fraction (%)
Weeks after Sham
C
Saline-MI (n=10)
Wls-gRNA-MI (n=17)
P = 0.0044 P = 0.0063 P = 0.0001
Fractional shortening (%)
Weeks after MI
D
Saline-MI (n=10)
Wls-gRNA-MI (n=17)
P = 0.0031 P = 0.011 P = 0.0002
Ejection fraction (%)
Weeks after MI


E
Saline
Wls-gRNA
Ligation
Apex

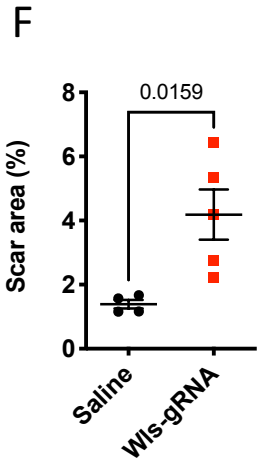

F
0.0159
Scar area (%)
Saline
Wls-gRNA

**Figure IV. *Wls* is required for neonatal heart regeneration.**

**A-D**, Fractional shortening **(A, C)** and ejection fraction **(B, D)** in control (saline-injected hearts) and *Wls-KD* (*Wls*-gRNA injected hearts) mice at different time points as indicated. **E**, Serial transverse heart sections at 11 weeks after P2 LAD-O, with quantification **(F)**. Scale bars=1 mm. Error bars represent ± standard error of the mean; **A-D** were determined by using determined by using the one-way ANOVA with Tukey's post hoc test, **F** were determined by using the Mann–Whitney test.

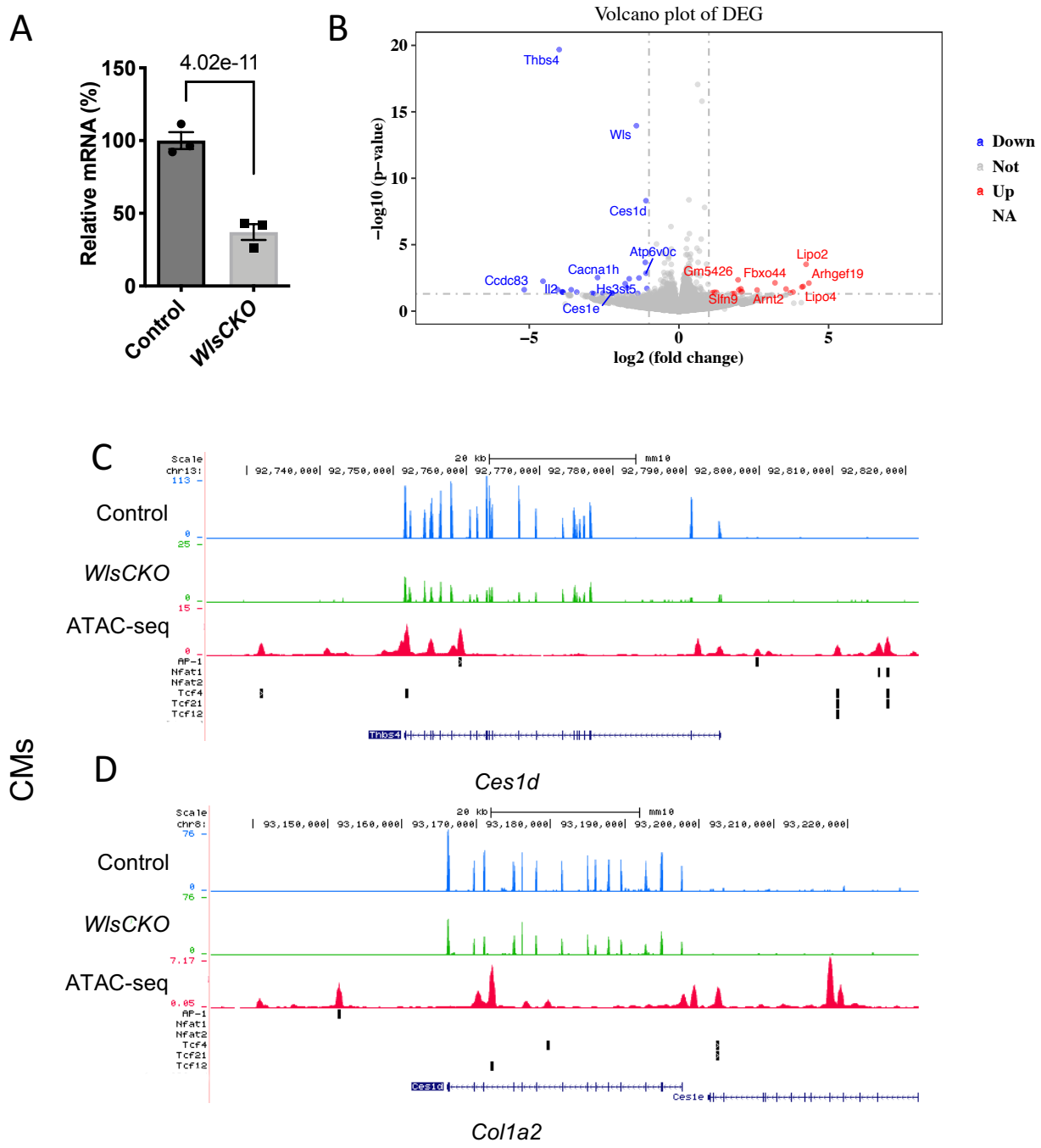



**Figure V. The expression of Wnt target genes in CMs.**

**A**, *Wls* expression in isolated CMs as determined by RNA-seq (n=3), statistics were showed as false discovery rate-adjusted p-value. mRNA expression levels are relative to the Control. **B**, Volcano plots show gene expression changes in isolated CMs from *Wls* CKO mice. The most changed genes are highlighted (n=3). **C**, **D**, Sequencing tracks for selected genes in CMs.

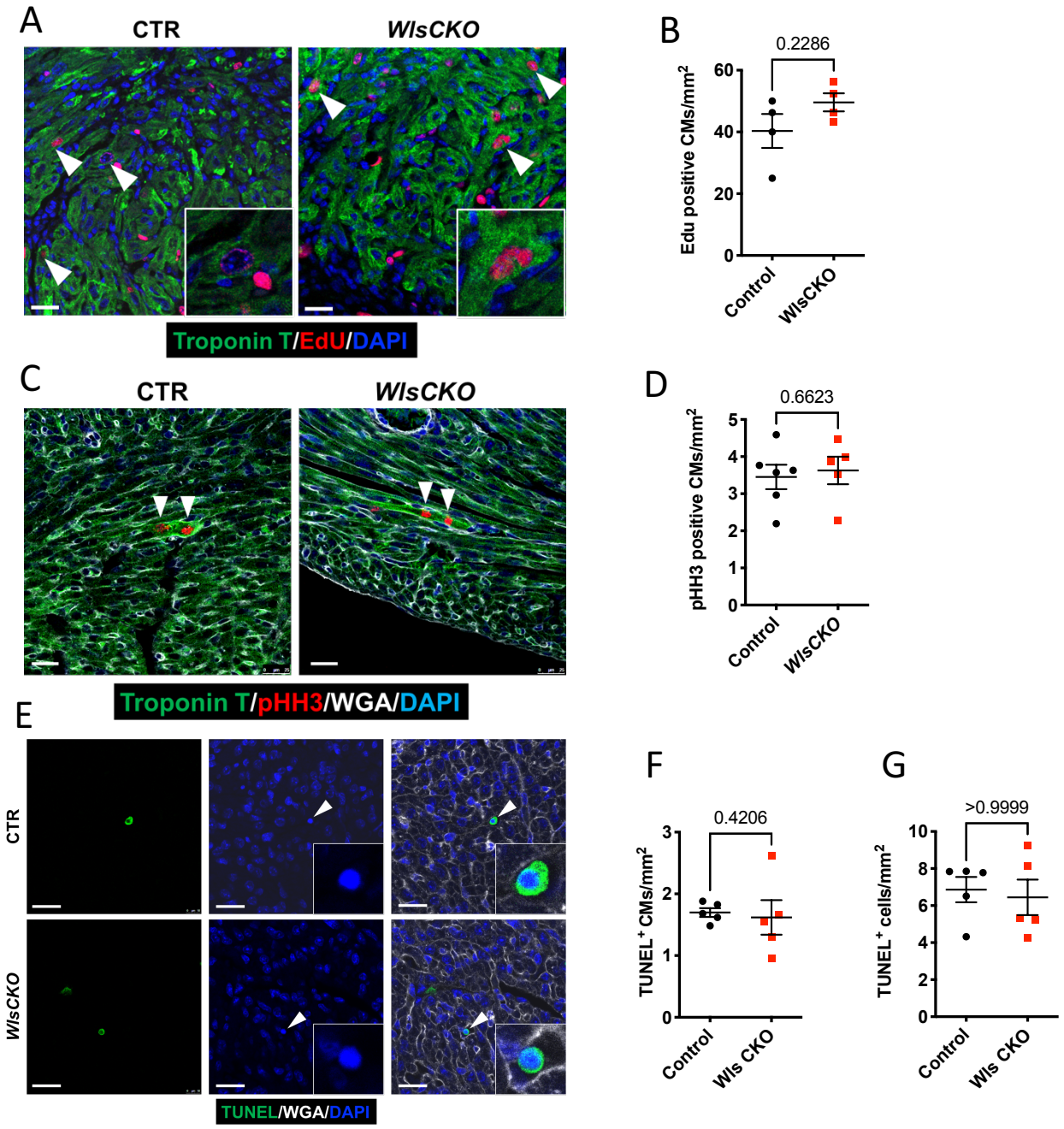



**Figure VI. *Wls* is dispensable for CM renewal after MI. A, B**, EdU staining reveals CMs in the cell cycle (red); heart sections were counterstained with the CM marker troponin T (green). Arrowhead indicates EdU-labeled CMs. **B**, Quantification of EdU-positive CMs (control, n=4; *Wls CKO*, n=4). Scale bars=25 μm. **C**, **D**, CMs in G2/M phase were labeled with pHH3 (red) and

troponin T (green); arrowhead indicates pHH3-positive CMs. **D**, Quantification of pHH3-positive CMs (control, n=6; *Wls* CKO*,* n=5). Scale bars=25 μm. **E**, TUNEL staining shows apoptotic cells (arrowhead). Scale bars=20 μm. **F, G**, Quantification of apoptotic CMs (CMs) and non-CMs (non-CMs), (control, n=5; *Wls CKO*, n=5). Error bars represent ± standard error of the mean; statistical test determined by using a Mann-Whitney test.

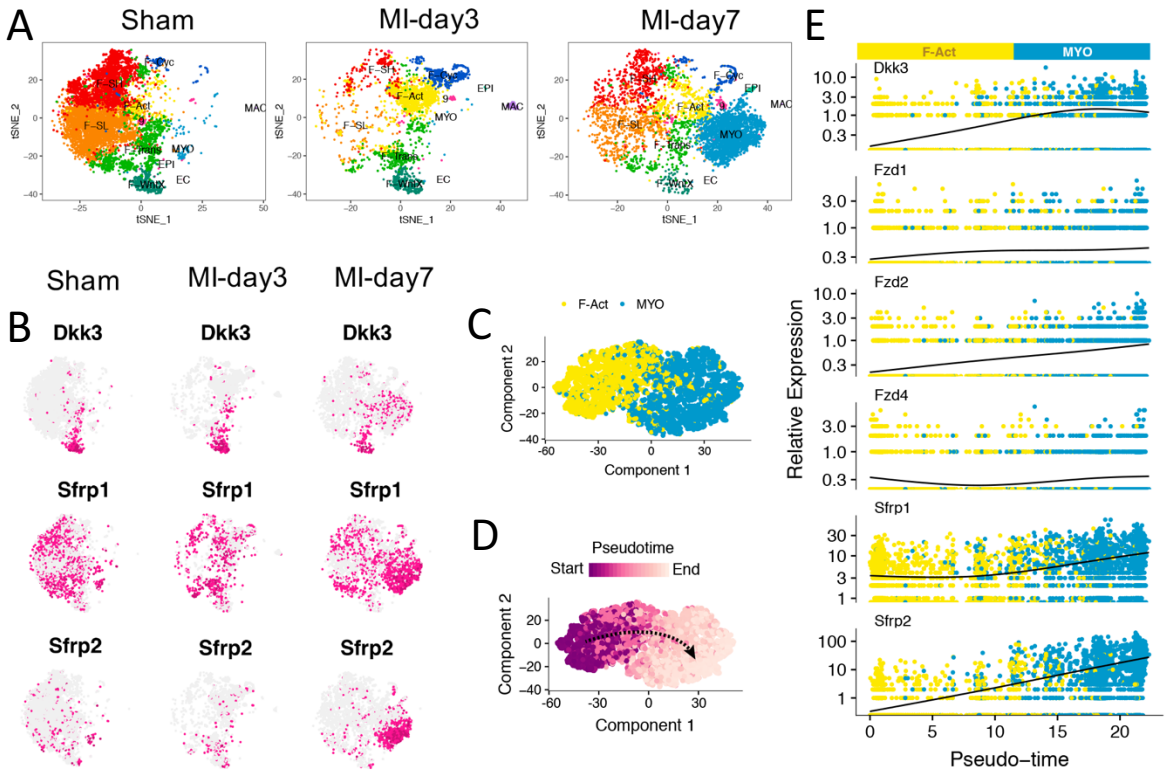



**Figure VII. Decreased Wnt signaling during fibroblast activation.**

**A**, tSNE plot of cardiac fibroblast clusters at different experimental time points. **B**, The expression of Wnt inhibitor genes in fibroblasts at different experimental time points. **C, D**, Pseudotime trajectory of activated fibroblasts (F-Act) and myofibroblasts (MYO). Arrow represents the F-Act to MYO differentiation trajectory. **E**, Expression and cell density plots of Wnt inhibitor and receptors genes across pseudotime. Points are each colored according to cluster identity from **(C)**.

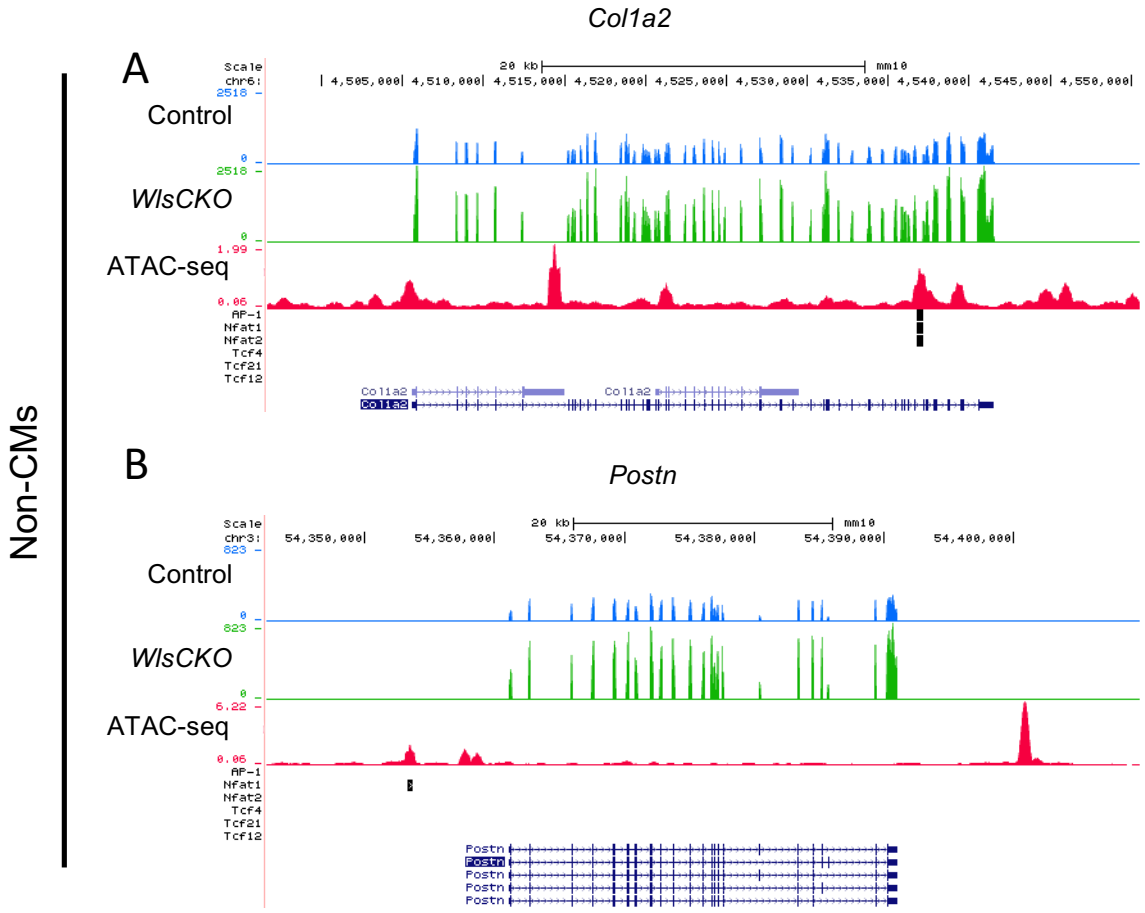



**Figure VIII. Expression of Wnt target genes in non-CMs.**

**A, B**, Sequencing tracks for selected genes in non-CMs.

**References**

1. Carroll KJ, Makarewich CA, McAnally J, Anderson DM, Zentilin L, Liu N, Giacca M, Bassel-Duby R and Olson EN. A mouse model for adult cardiac-specific gene deletion with CRISPR/Cas9. *Proc Natl Acad Sci U S A*. 2016;113:338-43.
2. Heallen T, Morikawa Y, Leach J, Tao G, Willerson JT, Johnson RL and Martin JF. Hippo signaling impedes adult heart regeneration. *Development*. 2013;140:4683-90.
3. Nascimento DS, Valente M, Esteves T, de Pina Mde F, Guedes JG, Freire A, Quelhas P and Pinto-do OP. MIQuant--semi-automation of infarct size assessment in models of cardiac ischemic injury. *PLoS One*. 2011;6:e25045.
4. Tao G, Kahr PC, Morikawa Y, Zhang M, Rahmani M, Heallen TR, Li L, Sun Z, Olson EN, Amendt BA and Martin JF. Pitx2 promotes heart repair by activating the antioxidant response after cardiac injury. *Nature*. 2016;534:119-23.
5. Morikawa Y, Heallen T, Leach J, Xiao Y and Martin JF. Dystrophin-glycoprotein complex sequesters Yap to inhibit cardiomyocyte proliferation. *Nature*. 2017;547:227-231.
6. Reynolds JO, Quick AP, Wang Q, Beavers DL, Philippen LE, Showell J, Barreto-Torres G, Thuerauf DJ, Doroudgar S, Glembotski CC and Wehrens XH. Junctophilin-2 gene therapy rescues heart failure by normalizing RyR2-mediated Ca(2+) release. *Int J Cardiol*. 2016;225:371-380.
7. Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M and Gingeras TR. STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*. 2013;29:15-21.
8. Love MI, Huber W and Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol*. 2014;15:550.
9. Croci O, De Fazio S, Biagioni F, Donato E, Caganova M, Curti L, Doni M, Sberna S, Aldeghi D, Biancotto C, Verrecchia A, Olivero D, Amati B and Campaner S. Transcriptional integration of mitogenic and mechanical signals by Myc and YAP. *Genes Dev*. 2017;31:2017-2022.