Yap Promotes Noncanonical Wnt Signals from Cardiomyocytes for Heart Regeneration

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Abstract

Rationale: During neonatal heart regeneration, the fibrotic response, which is required to prevent cardiac rupture, resolves via poorly understood mechanisms. Deletion of the Hippo pathway gene *Sav* in adult CMs increases Yap activity and promotes cardiac regeneration, partly by inducing fibrosis resolution. Deletion of Yap in neonatal cardiomyocytes (CMs) leads to increased fibrosis and loss of neonatal heart regeneration, suggesting that Yap inhibits fibrosis by regulating intercellular signaling from CMs to cardiac fibroblasts (CFs).

Objective: We investigated the role of *Wntless* (*Wls*), which is a direct target gene of Yap, in communication between CMs and CFs during neonatal heart regeneration.

Methods and Results: We generated two mouse models to delete Wls specifically in CMs (Myh6-Cas9 combined with *AAV9-Wls-g*RNAs, and *Myh6$^{cre-ERT2/+}$; Wls$^{flox/flox}$* mouse). Reanalysis of single-cell RNA-sequencing data revealed that Wnt ligands are expressed in CMs, whereas Wnt receptors are expressed in CFs, suggesting that Wnt signaling is directional from CMs to CFs during neonatal heart regeneration. *Wls* deletion in neonatal hearts disrupted Wnt signaling, showing as reduced noncanonical Wnt signaling in non-CMs. Four weeks after neonatal heart infarction, heart function was measured by echocardiography. *Wls* deletion in neonatal hearts after myocardial infarction impairs neonatal heart regeneration, marked by decreased contractile function and increased fibrosis. *Wls* mutant hearts display CF activation, characterized by increased extracellular matrix secretion, inflammation, and CF proliferation.

Conclusions: These data indicate that during neonatal heart regeneration, intercellular signaling from CMs to CFs occurs via noncanonical Wnt signaling to rebuild cardiac architecture after myocardial infarction.

Graphical Abstract
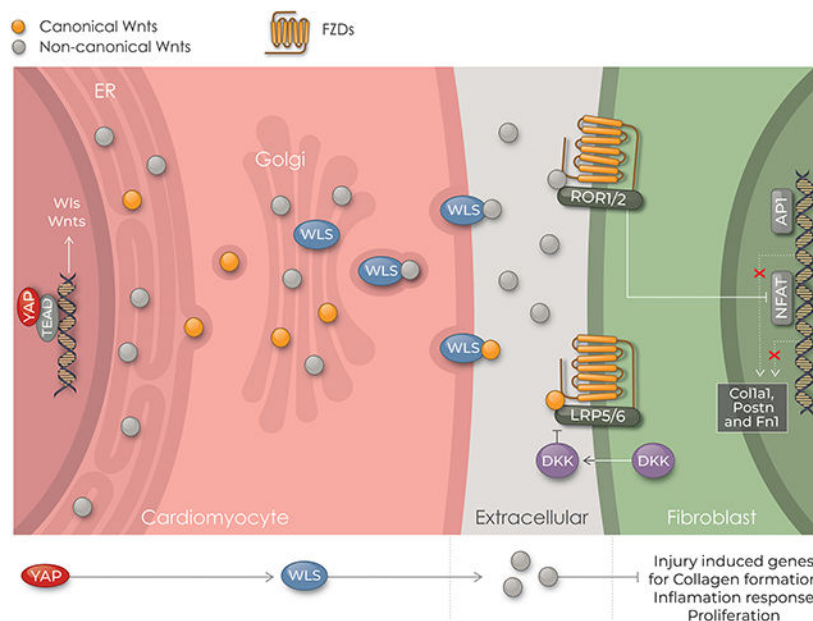
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INTRODUCTION

Signaling interactions between cells are fundamental to tissue development, homeostasis, and injury responses in multicellular organisms.[1] Some types of intercellular signaling require direct cell-cell contact that is mediated in large part through adhesion molecules, such as integrins, but also specialized membrane-bound receptor-ligand interactions such as Notch signaling. Other types of intercellular signaling occur distally, such as Wnt and Bmp-signaling that are fundamental to many organ systems including the heart.[2, 3] Introduction of tissue injury adds tremendous complexity to intercellular signaling via the recruitment of inflammatory cells that import multiple new signals to the injured organ to maintain both short- and long-term organ function. Although organ homeostasis and injury responses are highly complex, recent single-cell omics experiments have begun to uncover the molecular events of intercellular signaling.[4]

After myocardial infarction (MI), millions of cardiomyocytes (CMs) die in the adult mammalian heart and fail to regenerate, disrupting tissue architecture. To compensate for CM loss and prevent ventricular wall rupture, cardiac fibroblasts (CFs) undergo a cell state transition, proliferate, and differentiate into myofibroblasts, which deposit an extracellular matrix (ECM) and secrete factors that recruit inflammatory cells into the damaged heart.[5] In contrast to the adult heart, the neonatal mouse heart has transient regenerative ability shortly after birth[6–8] and total loss of regenerative ability 7 days after birth.[8,9] In addition to productive CM proliferation during neonatal heart regeneration, scar resolution allows for the recovery of pump function. However, mechanisms that regulate fibrosis resolution are poorly understood. Further insight into these mechanisms would have great medical value. In this study, we address whether Wnt-mediated intercellular signaling between CMs and CFs has a role in fibrosis resolution.

The Hippo pathway inhibits CM renewal and heart regeneration in the adult heart after MI.[10–12] In response to physiologic cues such as increased cell density, the conserved Hippo pathway kinase cascade phosphorylates the transcriptional cofactor Yap to inhibit Yap transcriptional activity.[13] When Hippo pathway activity is low, Yap enters the nucleus and forms a complex with TEA domain (Tead) transcription factors to induce the expression of genes that promote CM proliferation and heart regeneration.[11, 12, 14] In addition to activating CM proliferation, Hippo pathway inhibition enhances resolution of cardiac fibrosis in both the adult mouse heart after MI via poorly understood mechanisms. Hippo pathway mutants display increased CM proliferation, reduced fibrosis, and increased vascularity.[10, 11] In our previous studies, we specifically inhibited the Hippo pathway in CMs and observed the modulation of CFs. Thus, we hypothesized that the Hippo pathway regulates fibrosis through uncharacterized intercellular signaling between CMs and CFs.

Deletion of Yap in neonatal CMs after MI results in extensive fibrosis rather than neonatal heart regeneration, suggesting that uncharacterized Yap target genes, which likely encode signaling factors, promote the inhibition of fibrosis in neonatal heart regeneration.[15] The Wnt signaling pathway interacts closely with the Hippo pathway,[16] and multiple mechanisms connect the Hippo pathway to Wnt. For example, Yap has been shown to directly interact with the canonical Wnt effector β-catenin.[14] In addition, Yap interacts with the β-catenin destruction complex to modulate canonical Wnt signaling directly.[17] Moreover, other evidence potentially implicating Wnt-signaling in CM-CF signaling interactions include recent single-cell RNA sequencing (scRNA-seq) profiling which revealed the existence of CF subpopulations that express Wnt pathway genes, suggesting a role for Wnt signaling in the CF injury response.[18, 19]

The Wnt pathway, which includes nineteen Wnt ligands, is categorized into two subclasses: canonical Wnt/β-catenin signaling and β-catenin–independent, noncanonical signaling. Whereas canonical Wnt signaling relies on the transcriptional activity of β-catenin, noncanonical signaling, which encompasses the Wnt/planar cell polarity (PCP) and the Wnt/Ca^{2+} pathways, regulates gene expression via the transcription factors activator protein 1 (AP-1) and nuclear factor of activated T-cells (NFAT), respectively.[20–23] In the canonical pathway, Wnt ligands bind to the extracellular cysteine-rich domain of frizzled (FZD) receptors and LRP5/6 co-receptors to induce intracellular signal transduction. In the noncanonical pathway, Wnt ligands bind to FZD receptors and ROR1/2 co-receptors.[22] Here, we found that the Wnt trafficking gene *Wls* is a direct target gene of Yap in CMs. Furthermore, in the neonatal heart, noncanonical Wnt signaling that originates in CMs suppresses CF activation and promotes neonatal heart regeneration.

METHODS

Data Availability.

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.

RESULTS

Wls is a Direct Target Gene of Yap in CMs.

To identify the Yap-regulated factors that in turn regulate signaling from CMs to CFs, we screened for downstream Yap targets in CMs expressing Yap5SA, a version of Yap that contains serine-to-alanine mutations at Lats phosphorylation sites that confer enhanced transcriptional activity.[24] We reanalyzed chromatin immunoprecipitation sequencing (ChIP-seq) data, transpose-accessible chromatin with high-throughput sequencing (ATAC-seq) data, and RNA sequencing (RNA-seq) data from Yap5SA adult CMs and identified a Yap-regulated gene program that included the Wnt pathway (Figure 1A).[24] Expression levels of *Wls* and several Wnt ligand genes that are direct targets of Yap were upregulated

in Yap5SA CMs (Figure 1B, C). Several Tead motifs residing in accessible chromatin regions and enriched for the active chromatin marker H3K27Ac were found in the *Wls* locus (Figure 1C). We uncovered Tead motifs both upstream of the transcriptional start site of *Wls* and in the *Wls* gene body, supporting the notion that Yap uses Tead binding sites to direct *Wls* transcription. Furthermore, available Yap ChIP-seq data[25] revealed that Yap binds Tead elements in the *Wls* locus (Figure 1C). YAP activity is increased in CMs after MI,[26, 27] which may induce the expression of downstream targets of Yap to promote cardiac regeneration. Given that *Wls* is essential for outbound Wnt signaling by regulating the sorting and secretion of all Wnt ligands, we focused exclusively on *Wls* for further study.[28, 29]

We investigated the cardiac expression of *Wls* by using available RNA-seq data[30] and observed that *Wls* expression is expressed in regenerative-stage hearts in both CMs and CFs and that *Wls* expression is slightly reduced in nonregenerative-stage hearts (Figure 1D). Furthermore, available ChIP-seq data[31] revealed that levels of the active histone marker H3K27ac are reduced at the *Wls* locus in the P21-stage heart (Figure 1E). Collectively, these data suggest that *Wls* is a direct target of Yap and may be essential for neonatal heart regeneration.

Expression of Wnt Pathway Genes in the Heart.

To identify the Wnt ligand gene(s) that are expressed in CMs, we reanalyzed cardiac expression data from previously published RNA-seq datasets.[11, 24, 30, 31] Bulk RNA-seq data collected from mouse CMs at different timepoints revealed that a subset of Wnt ligand genes including *Wnt5a*, *Wnt5b*, *Wnt9a*, *Wnt11*, and *Wnt2b* was consistently expressed in CMs at higher levels than other Wnt ligands (Figure 2A). To further explore cardiac Wnt ligand gene expression, we reanalyzed previously published scRNA-seq data from embryonic (E) 14.5 hearts[32] and single-nuclear RNA-seq (snRNA-seq) data from P6 hearts (Figure 2B,C and Figure IA–D in the Data Supplement).[33] Notably, at both stages, *Wls* was expressed in numerous heart cell types, including CMs, CFs, endothelial cells, and macrophages (Figure 2B, C; Figure IA, B in the Data Supplement). At E14.5, CMs expressed *Wnt5a*, *Wnt11*, *Wnt16*, and *Wnt4* (Figure IA, B in the Data Supplement). Several Wnt ligand genes including *Wnt5a*, *Wnt5b*, *Wnt11*, and *Wnt9a* were expressed in P6 CMs, consistent with the bulk RNA-seq data (Figure 2A–C). These findings indicate that postnatal regenerative-stage CMs express both *Wls* and Wnt ligand genes. In addition, we observed that many Wnt ligand genes, such as *Wnt5a*, that were expressed in CMs are Wnts that function in the noncanonical pathway,[34–36] suggesting that noncanonical Wnt signaling is important for neonatal heart regeneration (Figure 2B, C; Figure IA, B in the Data Supplement).

To identify Wnt-responsive cells, we examined the expression of Wnt receptor genes *Fzd1-Fzd10* in both E14.5 and P6 datasets. At E14.5 *Fzd1, Fzd2,* and *Fzd3* were expressed in most cell types, whereas *Fzd1* and *Fzd2* were expressed in CMs and CFs and *Fzd3* was more restricted to CFs (Figure IC in the Data Supplement). In the P6 heart, *Fzd1*, *Fzd2*, *Fzd4*, and *Fzd8* were expressed in CFs. Additionally, at the P6 stage, Fzd receptor genes were also expressed in other cell types (*e.g. Fzd4* was also expressed in endothelial cells, and *Fzd2* in CMs) (Figure 2B, C). Approximately 42% of *Fzd2*-positive cells were CFs, and

Fzd2 expression levels were highest in CFs compared with other cell populations (Figure. 2B, C; Figure IIA in the Data Supplement). Interestingly, expression of several Wnt inhibitor genes including *Dkk3*, *Sfrp1*, and *Sfrp2* was also enriched in CFs at E14.5 and P6 (Figure 2B, C; Figures ID and IIB in the Data Supplement). Wnt inhibitors are secreted matricellular proteins that antagonize Wnt signaling via distinct mechanisms. Although sFRPs interact with all Wnt ligands to block signal transduction, DKK2 and DKK3 inhibit canonical Wnt signaling by specifically interacting with and preventing LRP5/6 activation. Together, these results suggest that Wnt signaling to CFs is tightly regulated. Experiments in mice in which Wnt inhibitor function was modulated support the notion that Wnt signaling must be tightly regulated for normal cardiac homeostasis and in response to injury.[37–42]

Wls Deletion in CMs Using CRISPR/Cas9 Gene Editing.

To functionally test the role of directional Wnt signaling from CMs to CFs *in vivo*, we inactivated *Wls* in CMs using CRISPR/Cas9 gene editing in a mouse line that expresses *Cas9* in CMs (*Myh6*-Cas9, referred to as *CM-Cas9*) (Figure IIIA in the Data Supplement).[43] Two guide RNAs (gRNAs) with specificity for exon 2 of *Wls* were delivered via adeno-associated virus 9 (AAV9) infection to regenerative-stage CMs (Figure IIIA in the Data Supplement). After a single injection of *AAV9-Wls*-gRNA into P3 *CM-Cas9* mice, approximately 70% of CMs were infected by AAV9, whereas more than 90% of CMs expressed *Cas9* (Figure 3A). CMs isolated from *CM-Cas9* mice injected with *AAV9-Wls*-gRNA exhibited gene editing at the *Wls* locus (Figure 3B). Consistently, we detected a 60% reduction of *Wls* expression in CMs from *CM-Cas9* mice injected with *AAV9-Wls*-gRNA compared with nontransgenic controls (Figure 3C, D). Immunofluorescence staining revealed a mosaic pattern of Wls expression in control hearts, with some CMs expressing higher levels of Wls (Figure 3E, and Figure IIIB in the Data Supplement). In hearts of *CM-Cas9* mice injected with *AAV9-Wls*-gRNA, Wls expression was globally decreased; hereafter, we refer to these mice as *Wls-knockdown (KD)* mice. Together, these results indicate that delivery of *AAV9-Wls*-gRNA into *CM-Cas9* mice is a rapid and efficient way to delete *Wls* specifically in CMs.

CM-specific Wls Deletion Disrupts Outbound Noncanonical Wnt Signaling.

We next sought to determine whether CMs and non-CMs respond to reduced Wnt signaling from CMs via an autocrine or paracrine mechanism(s), respectively. During Wnt signal transduction, canonical Wnt ligands directly induce the phosphorylation of the LRP5/6 co-receptor, whereas noncanonical Wnt ligand/receptor interactions induce phosphorylation of ROR1/2 and RYK.[20, 44, 45] We examined the phosphorylation of LRP6 (P-LRP6) and ROR1 (P-ROR1) in isolated CMs of *Wls-KD* mice. Total protein levels of both LRP6 and ROR1 were decreased in CMs after *Wls* deletion, consistent with previous reports that Wnt signaling stabilizes LRP6 and ROR1[46, 47] (Figure 4A, B). In addition, levels of both P-LRP6 and P-ROR1 (super-shifted bands) were decreased in CMs after *Wls* deletion. This indicated that both canonical and noncanonical signaling was reduced in CMs after *Wls* deletion in CMs and suggested an autocrine Wnt signaling loop in CMs (Figure 4A, B). Moreover, we observed decreased expression of β-catenin and its target Axin2 in *Wls*-deleted CMs, providing further evidence that canonical Wnt signaling is reduced in *Wls-KD* CMs (Figure 4A, B).

To validate our CRISPR data, we generated $Myh6^{cre-ERT2/+}$; $Wls^{flox/flox}$ conditional knockout (*Wls* CKO) mice, in which a tamoxifen (TAM)-inducible Cre recombinase is driven by the CM-specific promoter *Myh6*. Consistent with our *Wls-KD* data, we observed that LRP6 and ROR1 expression was decreased in CMs after TAM injection, indicating that autocrine canonical and noncanonical Wnt signaling in CMs is disrupted in *Wls* CKO hearts (Figure 4C, D). We then performed Western blot analysis of non-CMs, which included CFs, endothelial cells, and smooth muscle cells. Interestingly, we observed decreased levels of ROR1 and p-ROR1 but not of LRP6 or p-LRP6 in non-CMs (Figure 4E, F). To validate these data, we used a reporter mouse that detects canonical Wnt signaling (Tcf/Lef:H2B-GFP). Expression of the H2B-GFP reporter and nuclear β-catenin were both reduced in CMs but not in non-CMs of *Wls* CKO mice (Figure 4G–I). Together, these results suggest that whereas CMs respond to both canonical and noncanonical Wnt signaling, non-CMs respond only to noncanonical Wnt signaling originating from CMs.

Both *Wls-KD* and *Wls* CKO mice are healthy and phenotypically indistinguishable from wild-type controls up to four months of age. Cardiac contractile function in 3-month-old mice measured by using echocardiography revealed that *Wls-KD* mice exhibit cardiac contractility similar to that of controls (Figure IIIC, D in the Data Supplement). In addition, Masson's trichrome staining revealed that cardiac structure in *Wls-KD* mice is indistinguishable from that of controls (Figure IIIE in the Data Supplement). Thus, our findings indicate that reducing *Wls* activity in CMs does not affect postnatal cardiac homeostasis.

Wls is Required for Neonatal Heart Regeneration.

To determine whether *Wls* is required for neonatal heart regeneration after MI, we performed left anterior descending artery occlusion (LAD-O) in *Wls-KD* mice at P4, one day after the injection of *AAV9-Wls*-gRNA. No cardiac contractile function deficiencies were detected in control or *Wls-KD* sham-treated mice four weeks after treatment (Figure 5A, B and Figure IVA, B in the Data Supplement). Four weeks after LAD-O, cardiac contractile function in *Wls-KD* mice was significantly reduced compared with that in saline-injected controls (Figure 5A, B). Consistently, *Wls-KD* mice continued to exhibit decreased contractile function compared with saline-injected controls at six and eight weeks after LAD-O (Figure IVC, D in the Data Supplement). Moreover, we observed increased scarring and fibrosis in *Wls-KD* hearts (Figure IVE, F in the Data Supplement). Similar to *Wls-KD* mice, *Wls* CKO mice exhibited reduced contractile function four weeks after P2 LAD-O (Figure 5C, D), as well as increased scar size and fibrosis eight weeks after MI (Figure 5E, F). These findings indicate that *Wls* in CMs is essential for neonatal heart regeneration.

Reduced Wls is Tolerated by Neonatal Cardiomyocytes.

To determine the gene expression changes that arise in CMs depleted of *Wls*, we performed RNA-seq analysis on CMs isolated from control and *Wls* CKO hearts two weeks after P2-stage MI. *Wls* expression levels in *Wls* CKO CMs were reduced by more than 60% compared with control CMs, indicating the efficient deletion of *Wls* in these CMs (Figure VA in the Data Supplement). Interestingly, only a small number of differentially expressed genes was identified in CMs isolated from *Wls* CKO and control mice, suggesting that the

primary defect after *Wls* deletion in CMs originates from non-CMs. The genes with the most significantly altered expression levels in *Wls* CKO CMs, in addition to *Wls* itself, included *Thbs4* and *Ces1d*, which both had decreased expression levels in *Wls* CKO CMs (Figure VB in the Data Supplement). Motif analysis of the *Thbs4* and *Ces1d* loci revealed several binding sites for TCF, AP1,[23] and NFAT[48] (Figure VC, D in the Data Supplement, and see Methods), suggesting that *Thbs4* and *Ces1d* are direct targets of both canonical and noncanonical Wnt signaling in CMs. Notably, however, we detected no significant differences in proliferation and apoptosis rates between control and *Wls* CKO CMs after MI, suggesting that during neonatal heart regeneration, a 60% reduction in *Wls* is tolerated by neonatal CMs (Figure VIA–G in the Data Supplement).

Expression of Wnt Inhibitors is Upregulated in Cardiac Fibroblasts after Myocardial Infarction.

To gain insight into Wnt signaling in CFs after injury, we reanalyzed available scRNA-seq data from injured hearts at 3 and 7 days after MI.[18] We observed that two main fibroblast populations, F-SL (cells with low expression of Sca represent secretory fibroblasts) and F-SH (cells with high expression of Sca represent cardiac colony-forming mesenchymal stromal cell-like fibroblasts), maintain homeostasis in the heart. These fibroblasts transition to an activated-fibroblast phenotype (F-Act) 3 days after MI and further differentiate into myofibroblasts (MYO) 7 days after MI (Figure VIIA in the Data Supplement). Our analysis revealed that expression of Wnt inhibitors such as *Dkk3*, *Sfrp1*, and *Sfrp2* are elevated in differentiated myofibroblasts (Figure VIIB in the Data Supplement). We plotted the pseudotime differentiation trajectory (Figure VIIC–E in the Data Supplement) and observed that the expression of *Dkk3*, *Sfrp1*, and *Sfrp2* increase during the F-Act to MYO transition (Figure VIIE in the Data Supplement). These findings suggest that, albeit transiently, CFs receive less Wnt signal during cell state transitions from F-Act to MYO after MI, suggesting that low Wnt expression is a hallmark of the MYO cell state. Together, these results suggest that the activation of CFs is accompanied by a decrease in the Wnt signaling received. Thus, we hypothesize that *Wls* deletion in CMs further decreased non canonical Wnt signaling to CFs and leads to CF activation.

Wls Deletion in Cardiomyocytes Promotes Cardiac Fibroblast Activation after Myocardial Infarction.

To evaluate the CF response to *Wls* deletion, we performed RNA-seq analysis on non-CMs isolated from control and *Wls* CKO hearts 2 weeks after P2-stage MI. In contrast to CMs, non-CMs from *Wls* CKO hearts showed significant changes in gene expression after MI compared with control hearts (633 genes significantly changed; FDR<0.05). Gene ontology analysis of non-CMs in *Wls* CKO hearts after MI revealed the downregulated expression of genes associated with metabolism, cytoskeleton organization, cytokine production, and calcium-mediated signaling (Figure 6A) and the significant upregulation of genes associated with extracellular matrix organization, response to wounding, and cell growth (Figure 6A). Genes associated with CF activation such as *Postn, Fn1, Mmp9, Tnf,* and *Osm*, as well as genes encoding collagen proteins such as *Col1a1*, *Col1a2*, *Col3a1*[49–52] were increased in non-CMs from *Wls* CKO hearts. These data indicate that CF activation is upregulated in *Wls* CKO hearts (Figure 6B). Motif analysis uncovered several AP1 and NFAT binding

motifs in the *Col1a2* locus and an AP1 binding motif in the *Postn* locus, suggesting that these genes are directly regulated by noncanonical Wnt signaling (Figure VIIIA, B in the Data Supplement). Consistent with data showing that the expression of Wnt inhibitor genes increases during CF activation (Figure VIIA–E in the Data Supplement), our RNA-seq results reveal that expression of Wnt inhibitor genes is increased in non-CMs of *Wls* CKO mice (Figure 6C). Together, these results suggest that noncanonical Wnt signaling from CMs to CFs inhibits fibrosis after MI.

To investigate CF activation in *Wls* CKO hearts, we examined proliferation and differentiation in CFs.[53] Immunostaining for CFs (vimentin) and cells in S-phase (EdU) revealed that the number of EdU-positive fibroblasts is significantly increased in *Wls* CKO hearts compared with control hearts (Figure 6D). Consistent with this finding, we detected more pHH3-positive fibroblasts in *Wls* CKO hearts than in control hearts (Figure 6E).

Postn is a marker of activated fibroblasts and has reported roles in tissue regeneration and wound healing.[54] RNA-scope staining in the border zone revealed a significant increase in the number of *Postn*-positive cells in *Wls* CKO hearts compared with control hearts (Figure 6F). Collagen production in the infarcted zone was also increased in *Wls* CKO hearts compared with control hearts, indicating an increase in fibrosis (Figure 6G). We also examined the proliferation of endothelial cells in both control and *Wls* CKO hearts; however, no difference was detected (Figure 6H). Together, these results reveal that *Wls* deletion in CMs promotes CF proliferation, CF differentiation, and collagen production.

Noncanonical Wnt5a and Wnt9a Suppress Genes Associated with CF Activation.

Consistent with the sc-RNA-seq data from uninjured mouse hearts, our RNA-seq analysis showed that *Wnt5a*, *Wnt9a*, and *Wnt7b* were the most highly expressed genes in CMs (Figure 7A). No differences in the expression of Wnt genes in CMs were detected between control hearts and *Wls* CKO hearts (Figure 7A). To examine how noncanonical Wnt ligands regulate CF activation, we treated human CFs with Wnt5a and Wnt9a, two of the most highly expressed Wnt ligands in CMs. Before Wnt5a or Wnt9a treatment, we pretreated human CFs with LGK-974,[55] a porcupine inhibitor, to block autocrine Wnt signaling from human CFs. Interestingly, our results showed that treatment with Wnt5a or Wnt9a suppressed the expression of genes including *Col1a1*, *Col1a2*, *Col3a1*, *Col15a1*, *Fn1*, and *Postn* (Figure 7B–G). Thus, these results indicate that noncanonical Wnt ligands Wnt5a and Wnt9a may directly inhibit CF activation.

DISCUSSION

The Hippo pathway genetically and functionally interacts with Wnt-signaling in multiple contexts. Yap and Taz have been reported to suppress the Wnt/β-catenin pathway by directly binding to either Dishevelled[17, 56, 57] or β-catenin.[58] Moreover, Wnt ligands such as Wnt5a/b and Wnt3a activate Yap/Taz, which in turn suppress canonical Wnt/β-catenin signaling by inducing the expression of Wnt repressors, such as *DKK1*, *BMP4*, and *IGFBP4*.[59] Moreover, our published results reveal that nuclear Yap cooperates with β-catenin to promote the expression of numerous genes involved in heart development.[14] This study provides a new mechanism for the interaction between Hippo and Wnt signaling

in the heart. We show for the first time that *Wls*, which encodes a conserved multipass transmembrane protein required for transporting Wnt proteins to the plasma membrane for secretion,[28, 29, 60–64] is a direct target of Yap in CMs and regulates noncanonical Wnt signaling between CMs and CFs. Furthermore, we show that the CM-specific deletion of *Wls* in the neonatal heart results in the loss of regenerative capacity concomitant with increased fibrosis after MI. These findings suggest a model in which Yap in neonatal heart regeneration positively regulates non canonical Wnt signaling to inhibit cardiac fibrosis (Figure 7H).

In the *Wls* CKO heart, which loses its regenerative capacity and acquires characteristics similar to those of the profibrotic adult heart, we observed the upregulation of Wnt inhibitor genes including *Sfrp1* in non-CMs. These findings suggest that the expression of Wnt inhibitors is a marker for the activated profibrotic myofibroblast. Our findings further indicate that noncanonical Wnt ligands from CMs suppress CF activation after injury. This is consistent with another study showing that AAV9-mediated *Wnt11* expression in CMs leads to reduced fibrosis and inflammation, although the cell types influenced were not determined.[65] A previous study showed that deletion of *Sfrp2*, which results in increased Wnt signaling in CFs, inhibits collagen deposition after injury.[42] Our findings are also in line with those of another study in which the CM-specific overexpression of *Sfrp1* in mice, similar to the CM-specific deletion of *Wls*, led to increased scar size and worsened heart function after injury.[41]

While our data support a model for outbound directional signaling from CMs to non-CMs, Wnt signaling in the heart is highly complex and is known to also involve autocrine signaling in CFs [66]. In cultured CFs, Sfrp2 treatment stimulated cardiac CF proliferation, adding another line of evidence that noncanonical Wnt ligands suppress CF activation.[67] We observed highly expressed Wnt inhibitors, which indicates that Wnt signaling is tightly regulated in these cells. CMs are much larger in size than CFs. Considering the distribution of cells in the heart, CFs are almost completely encompassed by CMs. Thus, the effect of paracrine signals from CMs on CFs could be substantial.

In contrast to our data, several studies have revealed the beneficial effects of antagonizing Wnt signaling after injury.[68–72] In these studies, inhibitors of Wnt signaling were administered systemically raising the possibility that non-cardiac effects were involved in those experiments. Ruling out non-cardiac effects of systemically administered Wnt inhibitors, such as the response of immune cells, will require more experiments. Moreover, given that Wnt signals can emanate from many additional sources such as inflammatory cells, endothelial cells, CFs and CMs, further study is needed to determine the roles of CM-secreted Wnt ligands during heart regeneration.

By using adult post-MI and sham Axin2 reporter mice, a canonical Wnt reporter mouse, Mizutani et al.[73] showed canonical Wnt-responsive cells were localized to epicardium and subepicardial space. In addition, they observed an increased number of Axin2-positive cells, of which only ~8% expressed α-SMA, suggesting that canonical Wnt signaling plays a role in differentiation of a small subset of epicardial cells into myofibroblasts. Consistent with these findings, Xiang et al.[74] showed that β-catenin activity in CFs is

increased after trans-aortic constriction (TAC), a model of increased afterload, and functions to facilitate cardiac fibrosis in the adult TAC model. Notably, our model holds that in neonatal heart regeneration, noncanonical Wnt signaling inhibits fibrosis. Since it is known that noncanonical Wnt suppresses canonical Wnt signaling,[75] we hypothesize that after MI in the context of neonatal heart regeneration, noncanonical Wnt signaling from CMs functions to inhibit canonical Wnt signals emanating from other cell sources, including autocrine Wnts, to suppress fibrosis. Since Wnt inhibitors are targets of canonical Wnt signaling, the reduction of noncanonical Wnt signaling may also explain the increased Wnt inhibitor expression in non-CMs in *Wls* CKO heart. [76, 77] Further experiments are required to address this hypothesis.

Interestingly, Wang et al.[78] found, using single-cell RNA sequence profiling in the neonatal heart and in vitro studies, that Wnt signaling is upregulated in epicardial cells after MI. However, MI-induced Wnt signaling was stage specific. They found that R-Spondin 1, an activator of canonical Wnt signaling, was specifically upregulated in P1 epicardial cells after MI, whereas the noncanonical Wnt5a was only upregulated in P8 epicardial cells after MI. Combined with our findings, the data from Wang et al. raise the possibility that epicardial derived Wnt signaling works together with CM derived noncanonical Wnt signals to finely modulate CF activation after MI.

Regarding the limitations, our study demonstrated there was no significant difference of proliferation and apoptosis between hearts from control and WlsCKO mice. Whether Wls regulates the contractile and conductive abilities of CMs is unclear. In particular, it was reported that noncanonical Wnt signaling regulate Ca^{2+} transients [22, 79, 80], and canonical Wnt signaling regulates genes associate cardiac electrophysiology such as *Gja1* and *Scn5* [81]. Also, Further studies are required to determine how the noncanonical Wnt regulates fibroblast activation through ROR1/2 receptors.

In conclusion, our study provides important evidence that noncanonical Wnt signaling inhibits CF proliferation, collagen formation, and inflammation in neonatal heart regeneration (Figure 7H). Hence, studying the role of Wls and Wnt ligands in an established animal model of heart failure should prove to be valuable. In a previous study, we showed that Hippo deficiency reversed systolic heart failure, promoted CM proliferation, enhanced vascularity, and reduced fibrosis.[11] Whether Wls plays a similar role in the adult heart warrants further study. Our firm evidence that outbound noncanonical Wnt ligands from CMs inhibit CF activation after injury has potential clinical implications and places us closer to developing a therapeutic strategy for treating fibrosis in heart failure patients.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Nonstandard Abbreviations and Acronyms:

ATAC-seq	transpose-accessible chromatin with high-throughput sequencing
CF	cardiac fibroblasts
CHIP-seq	chromatin immunoprecipitation sequencing
CKO	conditional knockout
ECM	extracellular matrix
GO	gene ontology
LAD-O	left anterior descending artery occlusion
MI	myocardial infarction
MYO	myofibroblasts
RNA-seq	RNA sequencing
scRNA-seq	single-cell RNA sequencing
snRNA-seq	single-nuclear RNA-seq
TAM	tamoxifen

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NOVELTY AND SIGNIFICANCE

What Is Known?

- Neonatal mouse heart retains regenerative ability, and Yap is required for cardiac regeneration in mice

- Hippo pathway inhibition promotes cardiac regeneration through stimulating cell autonomous cardiomyocyte proliferation in mice

- Cardiac-specific Hippo deletion reverses fibrosis via a non-cell autonomous mechanism in murine cardiac regeneration

What New Information Does This Article Contribute?

- Wls is a direct target of Yap in cardiomyocyte, and required for neonatal murine heart regeneration

- Cardiac-specific Wls deletion in mice results in cardiac fibroblast activation

- Murine cardiomyocytes communicate with cardiac fibroblasts through noncanonical Wnt signaling

The Hippo signal transduction pathway is a kinase cascade that restrains the activity of YAP, a transcriptional cofactor that promotes cell proliferation. Blocking the Hippo pathway specifically in murine cardiomyocytes promotes heart functional recovery after injury, not only by stimulating cardiomyocyte proliferation, but also by preventing fibrosis but the mechanism through which it inhibits fibrosis is unknown. Here we discovered that Wntless (Wls), which is essential for Wnt ligand secretion, is a direct Yap target gene in the murine cardiomyocyte. In neonatal mouse heart regeneration, when Yap activity is high, Wls regulates noncanonical Wnt ligands secretion in the cardiomyocyte, which signals to cardiac fibroblasts and suppresses its activation and transdifferentiation. This work uncovers a previously unknown connection between Hippo and Wnt pathways in cardiac regulation. Importantly, this study reveals how Hippo and Wnt pathways coordinate the crosstalk between cardiomyocytes and fibroblasts during neonatal murine heart regeneration. We also provide new insights into basic cardiac developmental biology, which have important implications in developing novel therapeutic approaches for regenerative medicine.
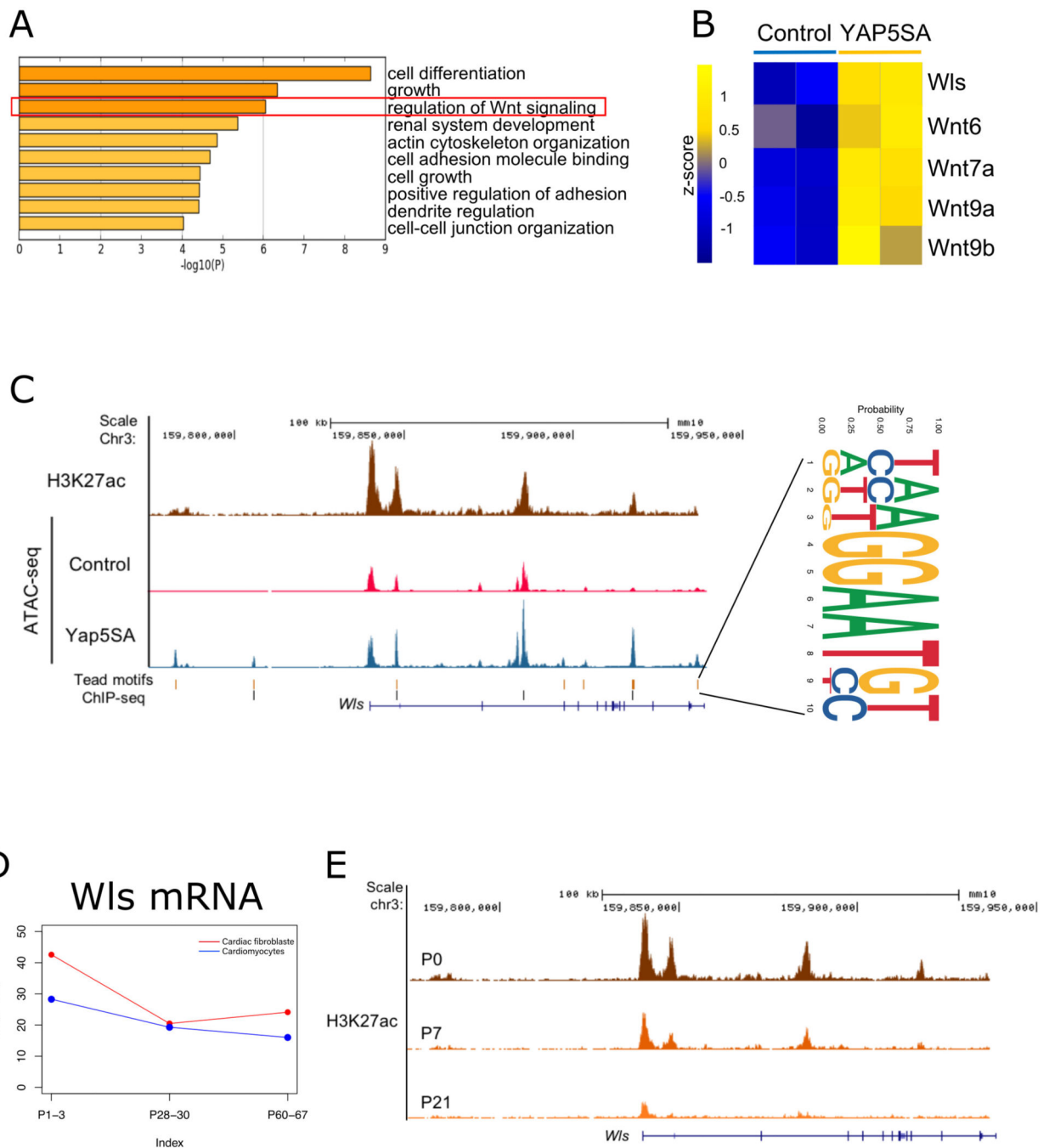
Figure 1. *Wls* is a direct target of Yap.

A, Gene ontology (GO) analysis of Yap5SA target genes (76 genes that overlapped in ChIP-seq, RNA-seq, and ATAC-seq datasets). The red square highlights genes involved in Wnt signaling regulation. **B**, Heatmap showing the upregulated expression of *Wls* and *Wnt* genes in Yap5SA CMs. **C**, H3K27ac ChIP-seq and chromatin accessibility mapping of the *Wls* locus by ATAC-seq. An increase in peaks (open chromatin) is seen in Yap5SA hearts. Tead motifs and enriched Yap ChIP-seq sites are also aligned. The consensus of 13 Tead motifs is shown on the right. **D**, *Wls* expression, as shown by RNA-seq. AU, arbitrary units;

RPKM, reads per kilobase of transcript per million mapped reads. **E**, H3K27ac ChIP-seq peaks mark heart-specific enhancers on the *Wls* locus at different developmental stages.
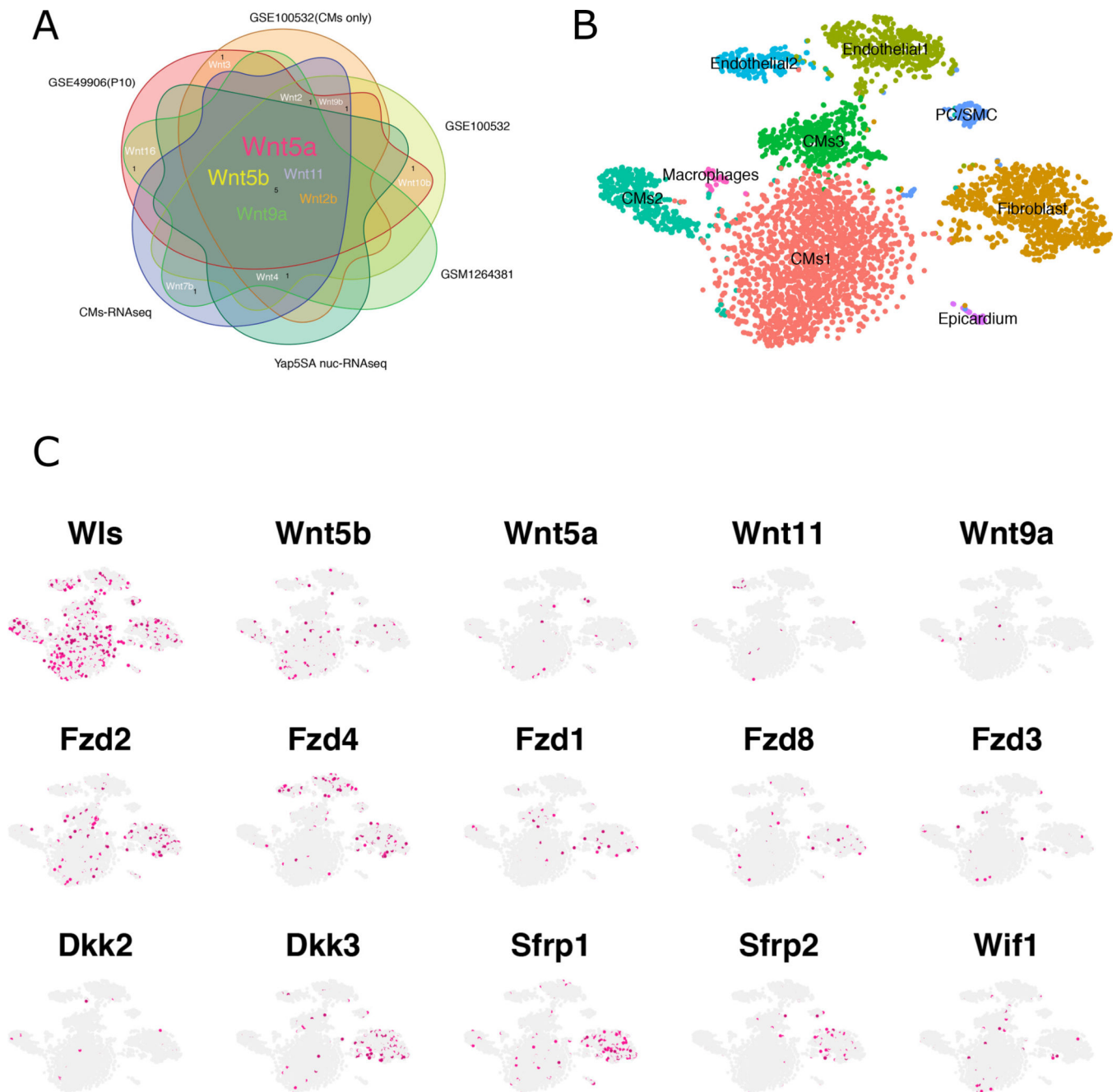
**Figure 2. Wnt gene expression in the heart.**
A, RNA-seq data from multiple sources reveal Wnt ligand expression in the heart. GSE100532, GSE49906, and GSE1264381 from available datasets. CM-RNAseq was performed on isolated WT CMs, and Yap5SA nucRNA-seq was performed on PCM1-enriched nuclei from Yap5SA overexpression heart. **B**, tSNE visualization of graph-based clustering and classification for P6 neonatal hearts. **C**, The expression pattern of Wnt components in P6 hearts.
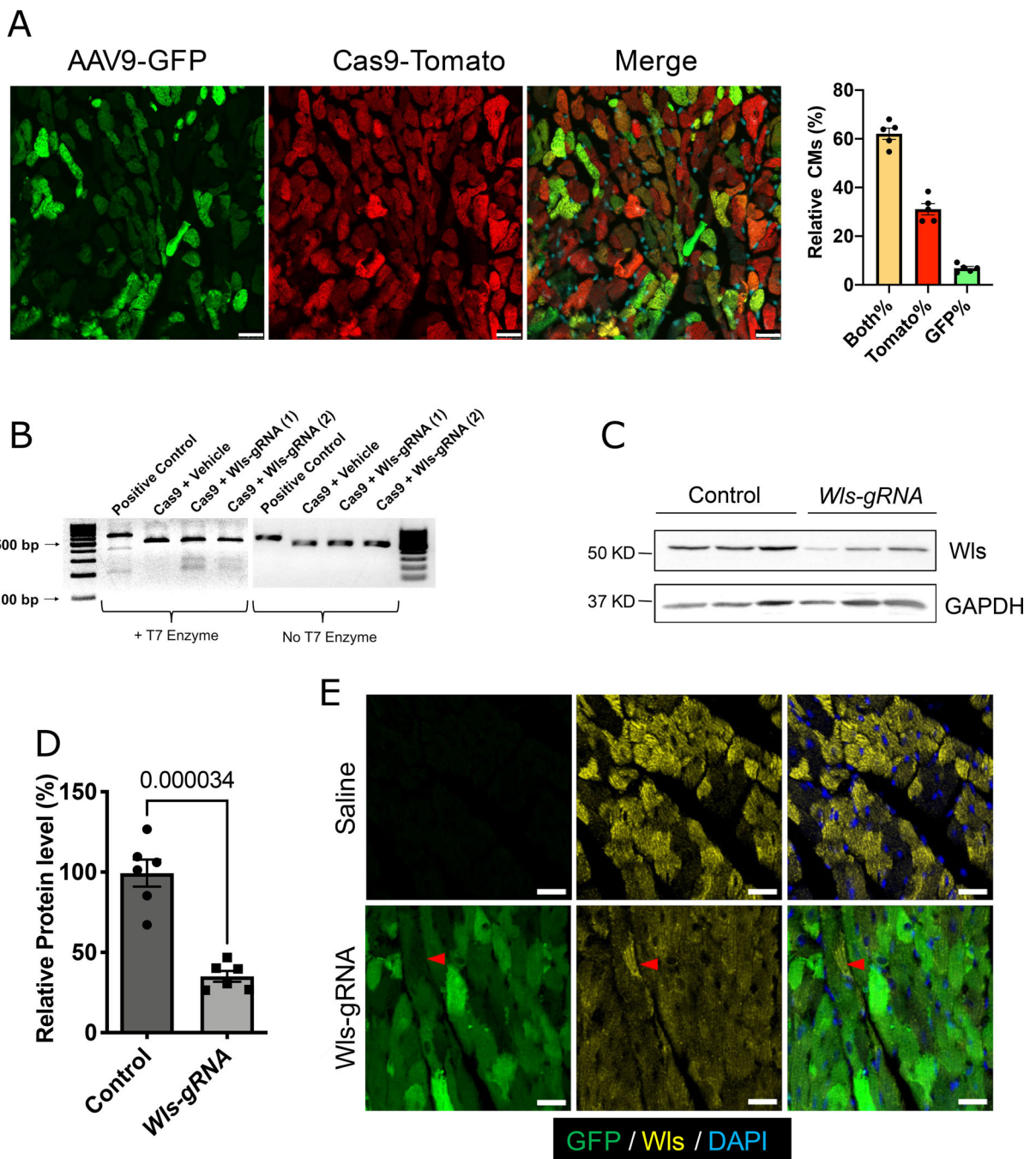
Figure 3. Deletion of *Wls* in CMs by CRISPR/Cas9.

A, Endogenous GFP (green, reporter of AAV9 infection) and tdTomato (red, reporter of Cas9) expression in *CM-Cas9* mice 10 weeks after *AAV9-Wls*-gRNA injection. Scale bars=25 μm. Bar graph shows the percentages of CMs with both tdTomato and GFP (Both%), tdTomato only (Tomato%), and GFP only expression (GFP%). **B**, T7 surveyor assay of isolated CMs. Cleaved bands indicate genome editing in exon 2 of *Wls*. (1) and (2) indicate samples from 2 different mice. **C**, Western blotting to detect Wls protein in isolated CMs. Quantification of Western blotting is shown in (**D**) (n=6). Protein expression levels

are relative to the GAPDH; the average value of the control group was set to 1. Error bars represent ± standard error of the mean. Mann-Whitney test was used for the comparison. Saline-injected wild-type littermates were used as controls. **E**, Immunofluorescence staining for Wls (yellow) in *Wls-KD* mice or saline-injected wild-type mice. Green (GFP), *Wls*-gRNA. White dashed line indicates uninfected cells that retain Wls expression. Saline indicates saline-injected wild-type littermates. Scale bars=25 μm.
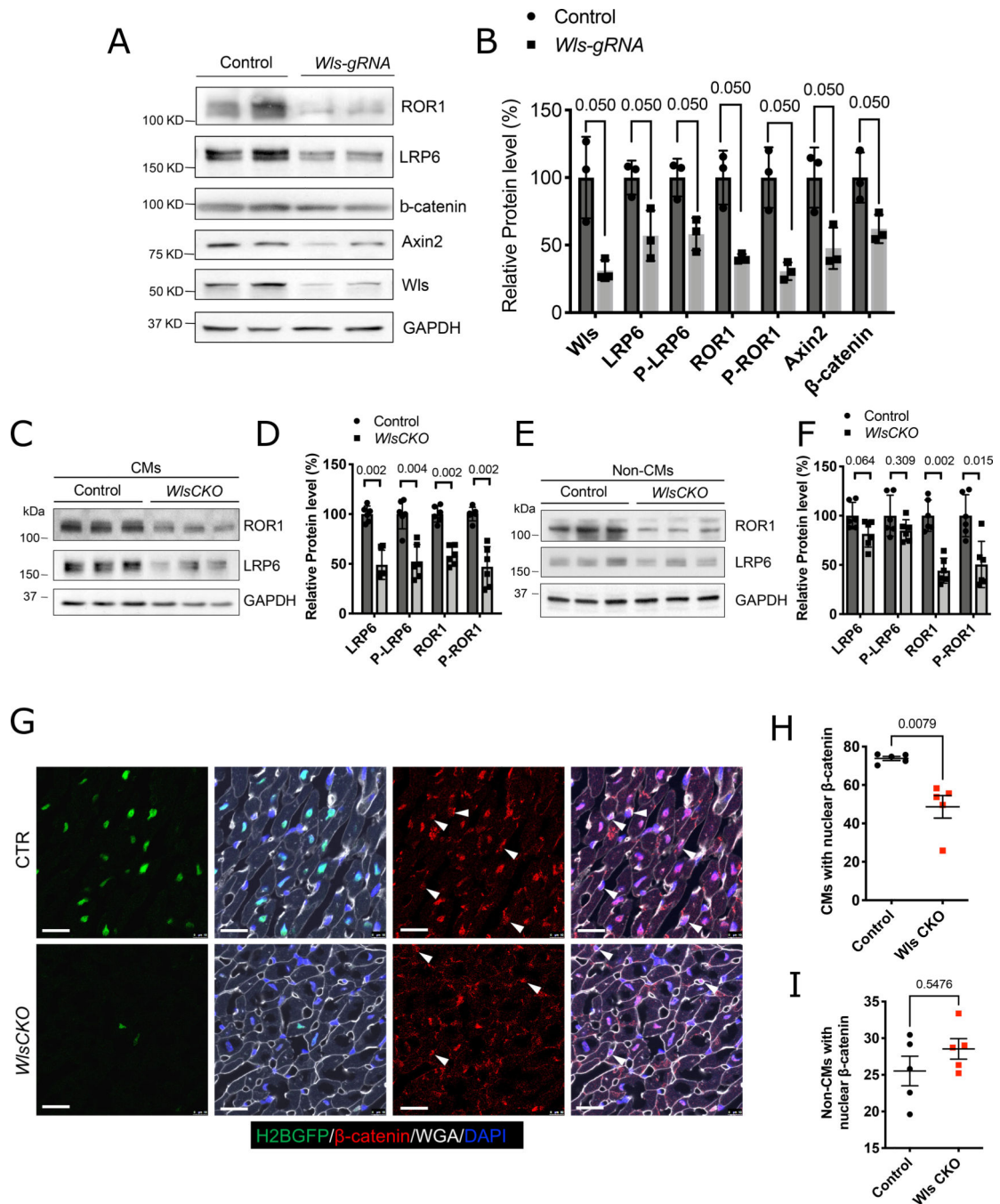
Figure 4. CM-specific *Wls* deletion disrupts Wnt signaling.
A, B, Immunoblotting of the indicated proteins in isolated CM lysates from *CM-cas9* mice
(A) with quantification **(B). C, D**, Western blotting of ROR1 and LRP6 proteins in isolated
CMs **(C)** with quantification **(D)**. l.e., long-exposure blot. **E, F**, Western blotting of ROR1
and LRP6 proteins in isolated non-CMs **(E)** with quantification **(F).** Littermate *Wls^{flox/flox}*
mice were used as controls (CTR). Western blotting of ROR1 and LRP6 consistently
detected two bands; the higher molecular weight bands are the phosphorylated proteins.
Protein expression levels are relative to GAPDH; the average value of the control group

was set to 1. **G**, Tcf/Lef:H2B-GFP reporter mice exhibited reduced activity of canonical Wnt/β-catenin signaling in CMs of *Wls CKO* mice. IF staining reveals decreased nuclear intensity of β-catenin in CMs of *Wls CKO* mice. Arrowhead indicates non-CMs with nuclear β-catenin. Scale bars=20 μm. **H, I**, Quantification CMs or non-CMs with nuclear β-catenin (n=5). Error bars represent standard error of the mean; **B**, **D, F, H, and I** were determined by using the Mann–Whitney test. Each dot represents a single animal.
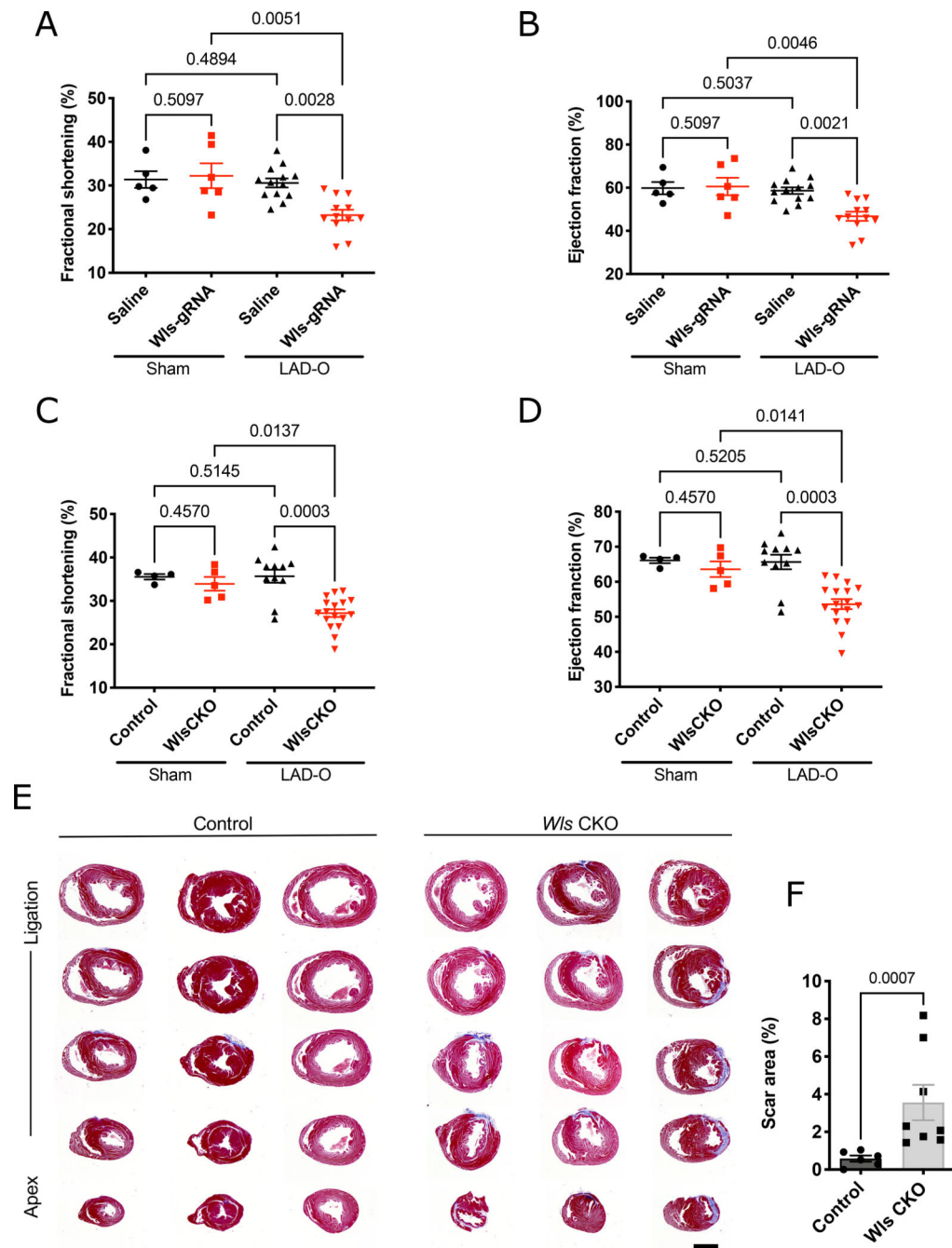
Figure 5. Requirement of *Wls* for neonatal heart regeneration.
A-D, Echocardiography revealed reduced fractional shortening **(A, C)** and ejection fraction **(B, D)** 4 weeks after P4 LAD-O in mice with the CRISPR/Cas-mediated deletion of *Wls* **(A, B)** or via the Cre/loxP-mediated conditional knockout of *Wls* (*Wls* CKO) **(C, D)** (each dot represents a single animal; n=5 for Saline-Sham; n=6 for Wls-gRNA-Sham; n=13 for Saline-LAD-O; n=12 for Wls-gRNA-LAD-O; n=4 for Control-Sham; n=5 for WlsCKO-Sham; n=11 for Control-LAD-O, n=17 for WlsCKO-LAD-O). Saline-injected wild-type littermates were used as controls for mice with CRISPR/Cas-mediated *Wls* deletion;

Myh6$^{cre-ERT2/+}$ mice were used as controls for *Wls* CKO mice. **E**, Trichrome-stained serial transverse sections of mouse hearts 8 weeks after LAD-O. **F**, Scar size quantification is shown. Error bars represent standard error of the mean; In A-D, the comparison were determined by using the Kruskal Wallis with two-stage Benjamini, Krieger, & Yekutieli FDR-based multiple comparison, F was determined by using the Mann–Whitney test. Scale bars=1 mm.
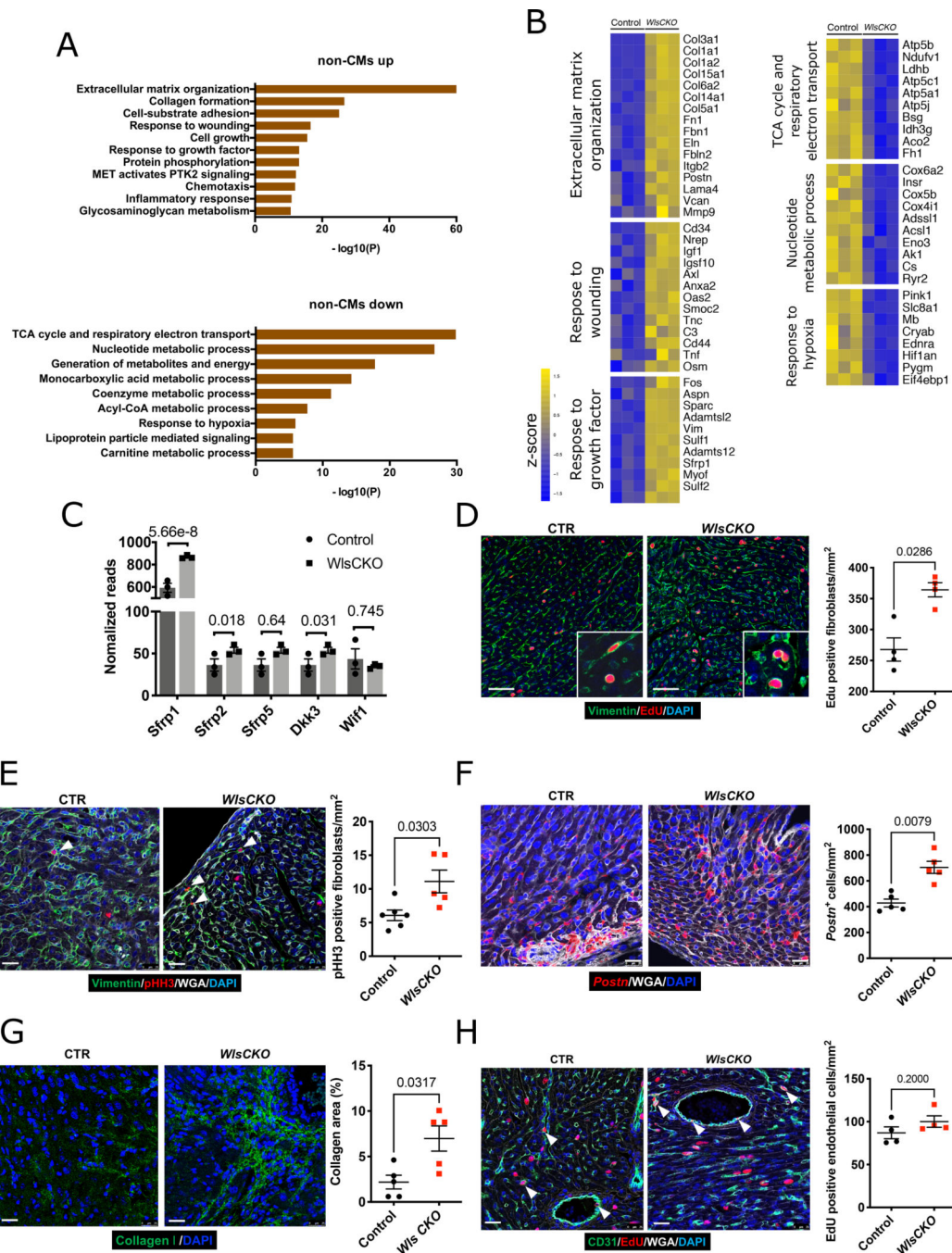
Figure 6. CM-specific deletion of *Wls* promotes CF activation after injury.
A, GO analysis of 298 downregulated and 335 upregulated genes in non-CMs of *Wls* CKO mice (n=3). **B**, Heatmap shows the expression of associated genes in non-CMs of *Wls* CKO mice. **C**, RNA-seq shows the expression of Wnt inhibitors in non-CMs of control and *Wls* CKO mice, statistics were showed as false discovery rate-adjusted p-value. **D**, EdU incorporation (red) reveals the increased proliferation of fibroblasts with vimentin as a marker (green) in *Wls* CKO mice. Littermate *Wls^flox/flox^* mice were used as the control (control, n=4; *Wls* CKO, n=4). Scale bars=50 μm. **E**, pHH3 immunofluorescence staining

(red) of G2/M fibroblasts (green) in *Wls* CKO mice (control, n=6; *Wls* CKO, n=5). Scale bars=25 μm. Mice were challenged with LAD-O at P2, and hearts were harvested one week later at P9 for **(D)** and **(E)**. **F**, RNA-scope reveals increased *Postn*-positive cells in *Wls* CKO mice (n=5 for each genotype). Scale bars=25 μm. **G**, Increased collagen formation in *Wls* CKO mice (n=5 for each genotype). Scale bars=25 μm. Mice were challenged with LAD-O at P2, and hearts were harvested two weeks later at P16 for **(F)** and **(G)**. **H**, CD31 and EdU staining of endothelial cell proliferation. Mice were challenged with LAD-O at P2, and hearts were harvested one week later at P9. Error bars represent the standard error of the mean; comparisons were determined by using the Mann–Whitney test.
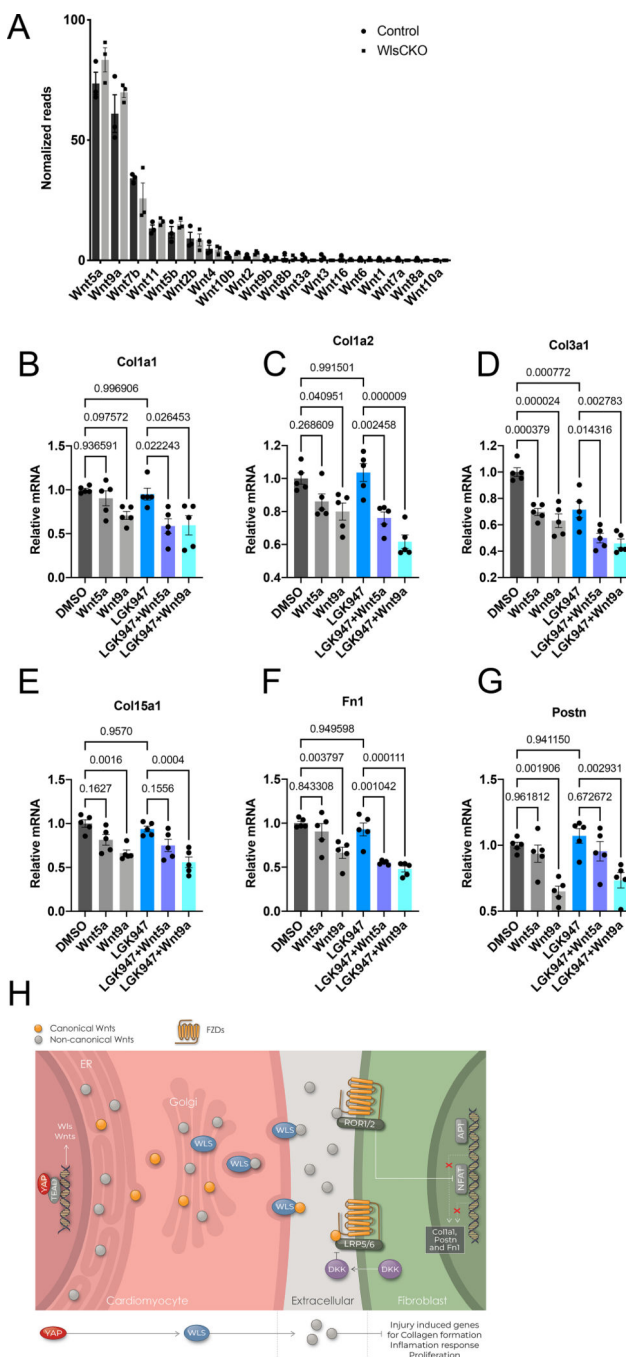
Figure 7. Noncanonical Wnt5a and Wnt9a suppress CF activation.
A, RNA-seq analysis of Wnt ligand gene expression in CMs from control and *Wls* CKO
mice (n=3). Littermate *Wls*^*flox/flox* mice were used as the control. **B-G**, qRT-PCR analysis
of gene expression in human CFs treated with Wnt5a or Wnt9a (both 100 ng/ml) for 24
hours with or without pretreatment with LGK-974 (1 μM) for 48 hours before harvesting
as indicated. One-way ANOVA with Dunnett's post hoc test was used for the comparison.
Data from n=5 independent batches of cells run in triplicate. mRNA expression levels are
relative to beta-actin; the average value of samples treated with DMSO was setting to 1. **H**,

Working model in which cardiomyocytes inhibit fibroblast activation through Wls-mediated noncanonical Wnt signaling during neonatal heart regeneration.