Supplemental Material

Conditional, tissue-specific CRISPR/Cas9 vector system in zebrafish reveals the role of neuropilin-1b in heart regeneration.

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Figure S1

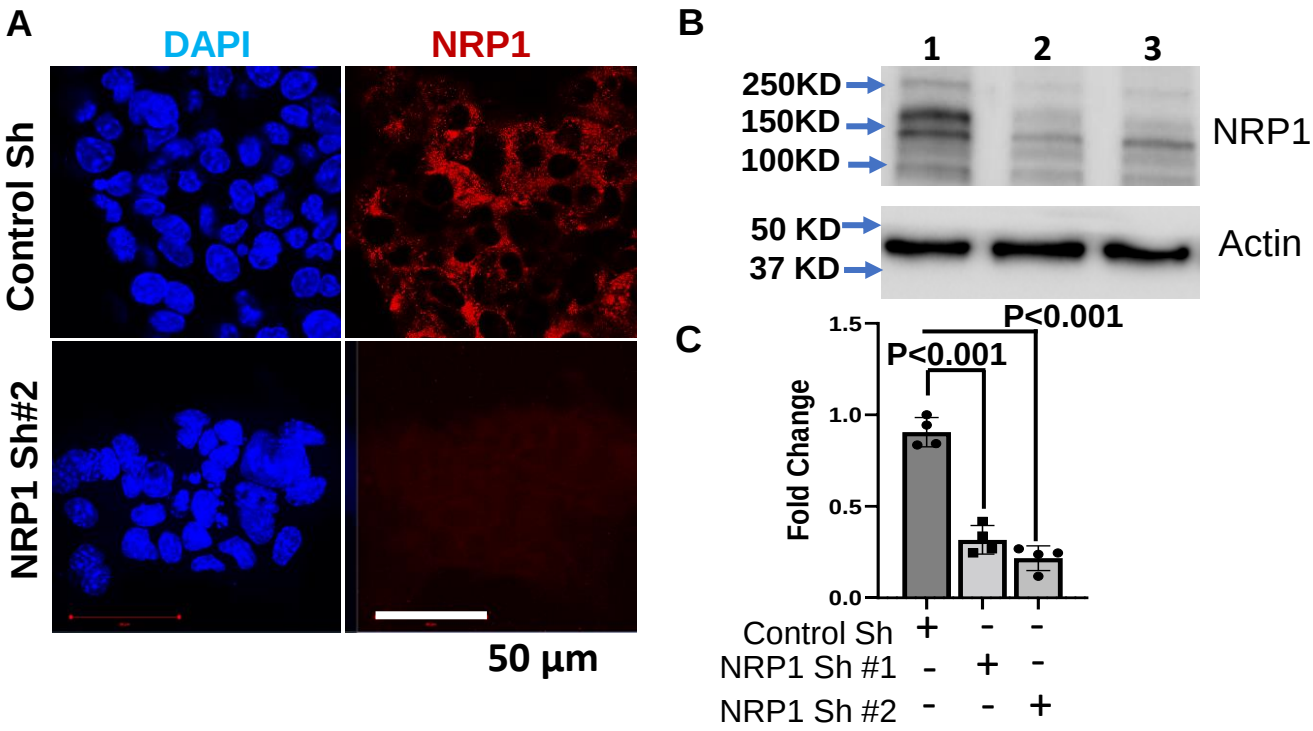


Figure S1: NRP1 antibody validation in Cardiomyocytes. (A). Immunofluorescence staining of Control shRNA and NRP1 shRNA treated mouse cardiomyocytes (HL-1) with NRP1 antibody (red) (Neuropilin-1 (D62C6) Rabbit mAb #3725). (**B)**. Western blotting showing the NRP1 downregulation in HL-1 cardiomyocyte infected with NRP1 shRNA #1 and NRP1shRNA #2. (**C)**. Quantification showing the fold change in protein expression from B. Error bars represents the mean ± SEM. The western experiment was repeated three times.. Statistical significance was evaluated with Prism 9.0 software by using Student's *t*-test. P values below 0.05 were considered significant and if lower as indicated.

Figure S2

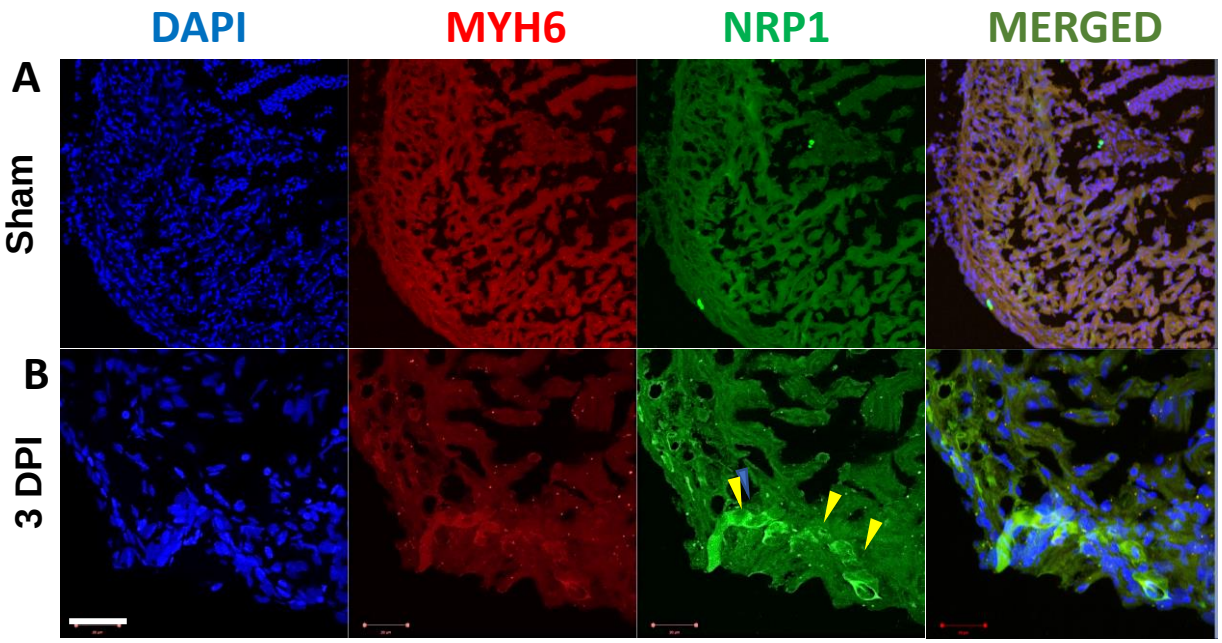


Figure S2: NRP1 is upregulated in the injured cardiomyocytes. **(A)** Confocal image showing NRP1 and MYH6 costaining in uninjured zebrafish ventricle cryosection and (**B)** Confocal image showing NRP1 and MYH6 costaining in zebrafish ventricle after injury (3 day after cryoinjury). Yellow arrowhead indicated the NRP1 over-expression at injury site. Scale = 20µm

Figure S3

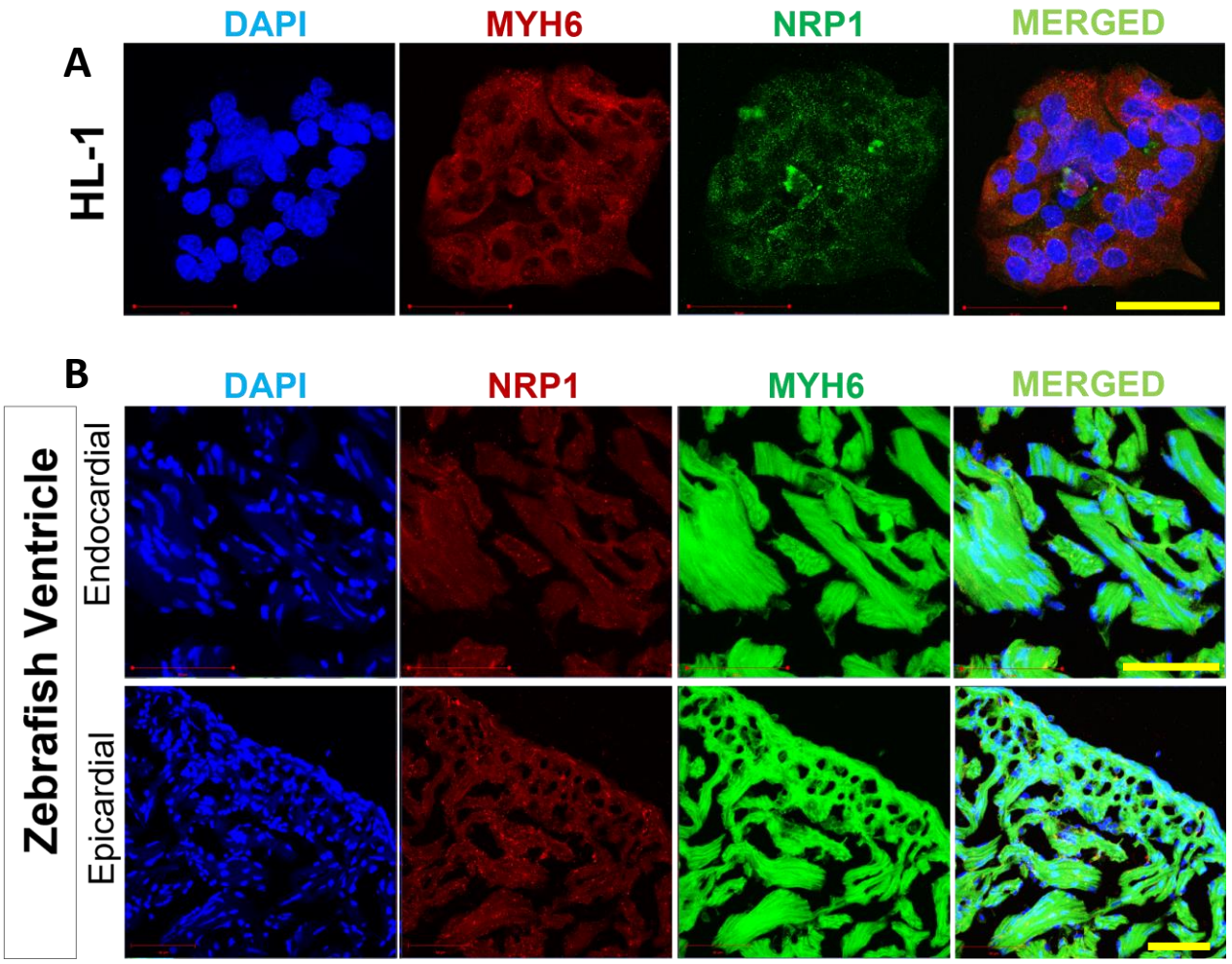


Figure S3: NRP1 expression in cardiomyocytes. (**A)**. Confocal image showing NRP1 and cardiomyocyte marker, MYH6 (Anti-MYH6 antibody [3-48] ab15) costaining in mice ventricle cardiomyocyte (HL-1). (**B)**. NRP1 and cardiomyocyte marker (MYH6) costaining in adult zebrafish ventricle. Scale = 50µm.

Figure S4

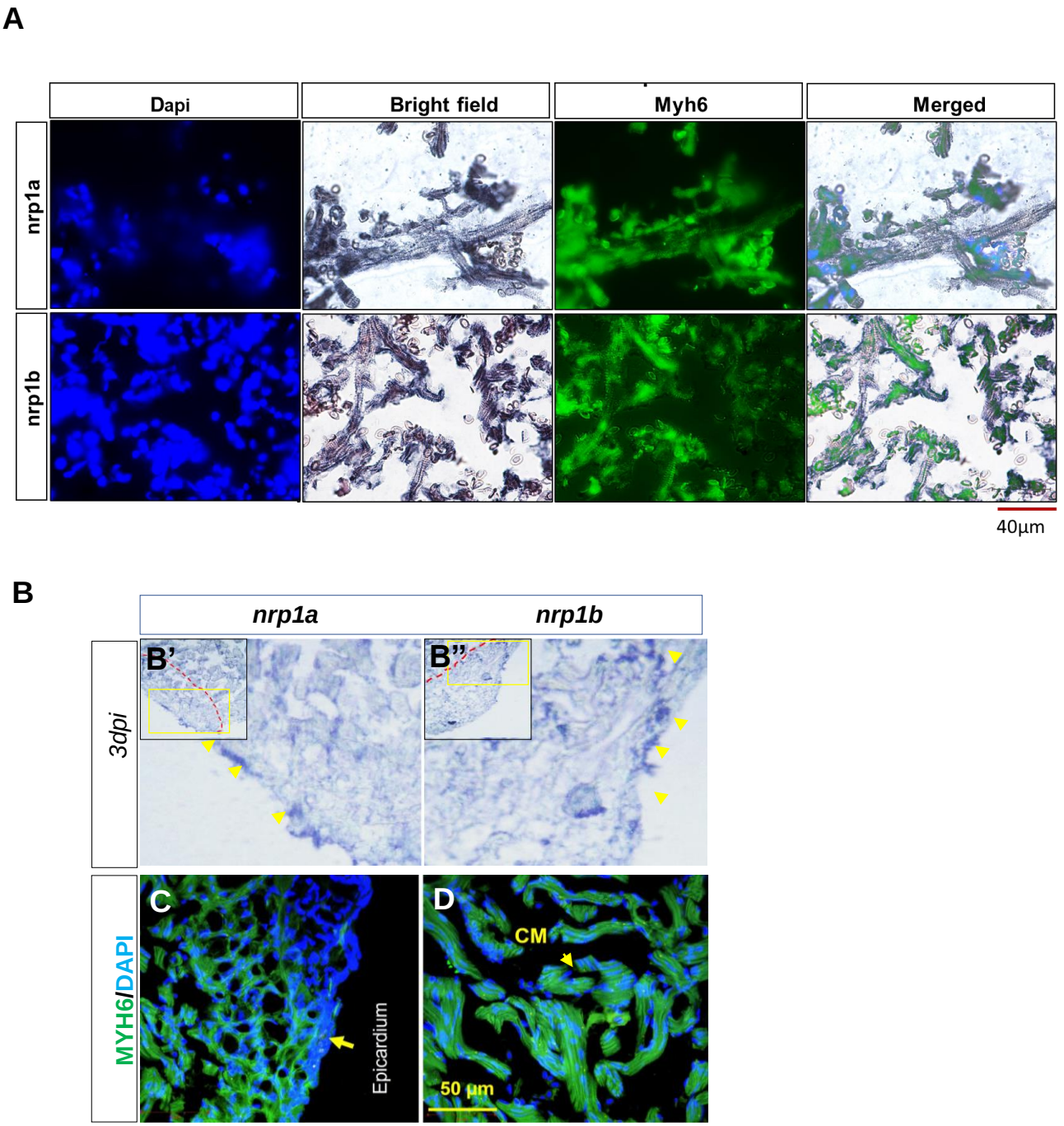


Figure S4: Cardiomyocytes specific expression of nrp1a and nrp1b in zebrafish. (A-B). In situ hybridization showing nrp1a expression in adult wild type heart ventricle section **A**. Representative image showing 6-month-old zebrafish ventricle sections double stained *for in-situ hybridization of* nrp1a and nrp1b expression (purple) and immunofluorescence-stained myosin heavy chain 6 (MYH6) expression in green. Nuclei are labeled with dapi (blue) **B**. nrp1a and nrp1b expression at injury site in adult wildtype heart ventricle (B'; nrp1a in situ hybridization showing nrp1a expression in heart ventricle section after 3 dpi, B"; nrp1b expression in heart ventricle section after 3 dpi. (**C-D)**. Confocal image of zebrafish ventricle cryosection stained with cardiomyocytes-specific myosin heavy chain 6 (green) and nuclear-DAPI. (C; Periphery and D; inner region). Image were captured by EVOS M5000 Microscope using 63x objective in A. Zeiss confocal microscope in C and D. Yellow arrowhead indicates the cardiomyocyte. Yellow arrow indicates epicardium. Yellow box indicate the enlarged area analyzed. N=5 images were analyzed for each group.

Figure S5

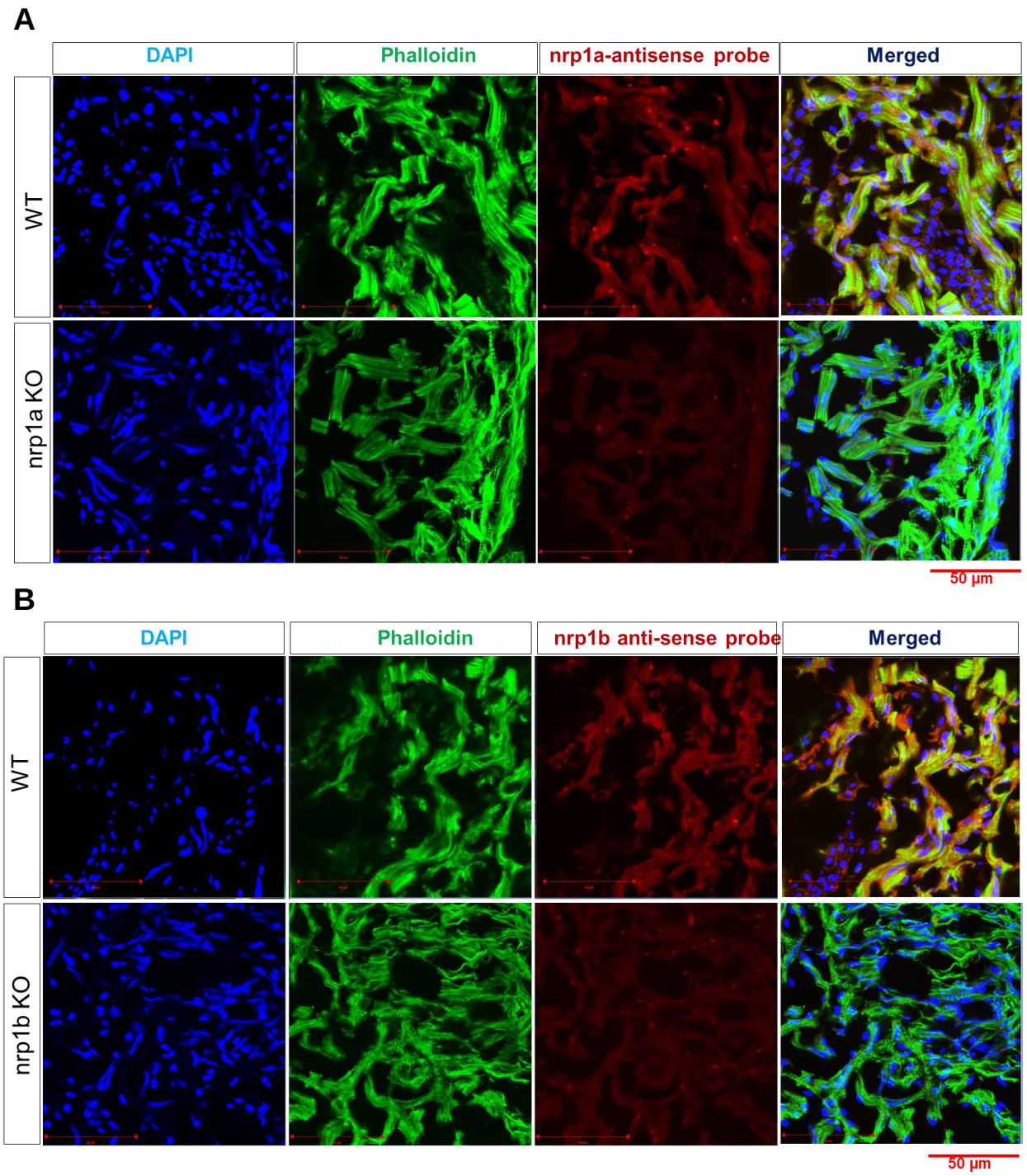


Figure S5: Representative Z-stack confocal image showing the Fluorescence *in situ (FISH)* staining of nrp1a and nrp1b in the CMs using antisense nrp1a and nrp1b specific probes labelled with Cy3 and CMs labelled with CM specific F-actin (Alexa - 488 - phalloidin) in Wt and nrp1a and nrp1b knocked out heart ventricle section. Images were captured by using Zeiss confocal microscope (LSM 880) using 63x objective. nrp1a and nrp1b in Red, F-actin in green, Nuclei in blue, Yellow : merged. Scale bar = 50µm. 5 images were analyzed in each group.

Figure S6

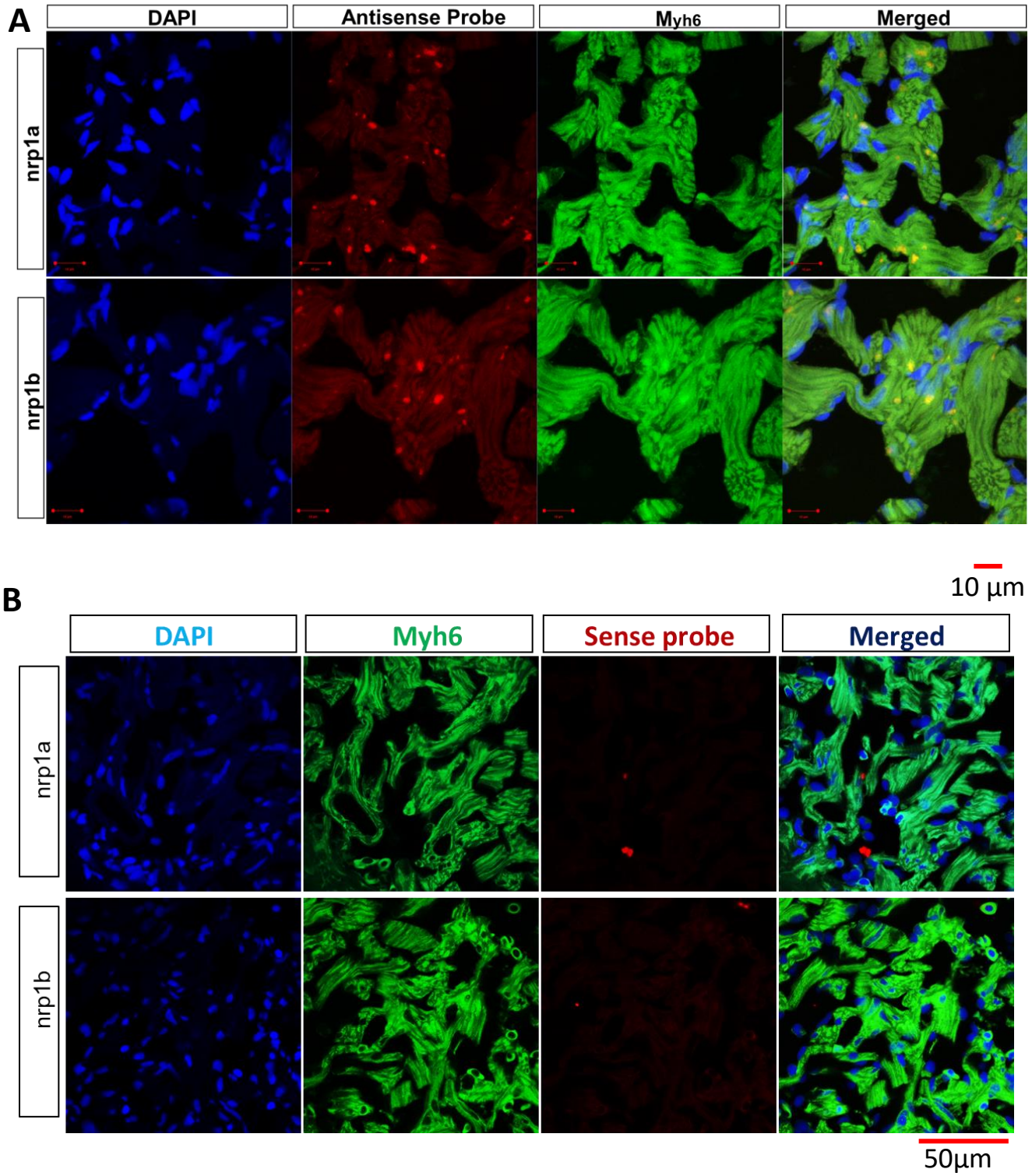
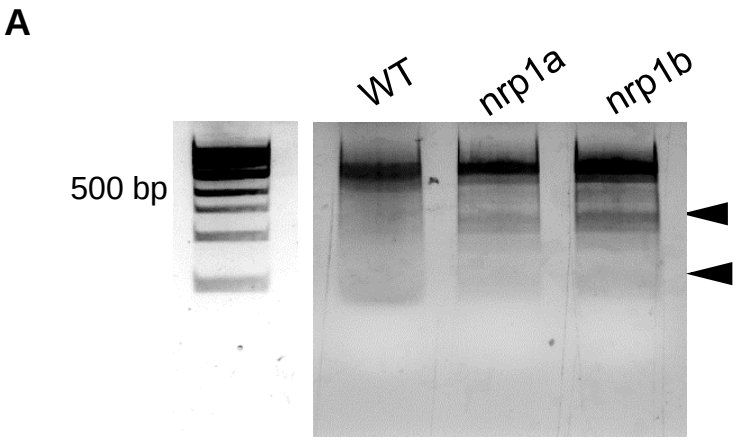


Figure S6: A. Representative Z-stack confocal image showing the Fluorescence *in situ (FISH)* staining of nrp1a and nrp1b in the CMs using antisense nrp1a and nrp1b specific probes labelled with Cy3 and CMs labelled with cardiac myosin heavy chain 6 (Myh6) in Wt heart ventricle section. **B.** Representative Z-stack confocal image showing the Fluorescence *in situ (FISH)* staining of nrp1a and nrp1b in the CMs using sense nrp1a and nrp1b specific probes labelled with Cy3 and CMs labelled with CM specific MYH6 in WT heart ventricle section. Images were captured by using Zeiss confocal microscope (LSM 880) using 100x objective in A and 63x objective in B. nrp1a and nrp1b in Red, Myh6 in green, Nuclei in blue, Yellow: merged. Red puncta in A represents nrp1a and nrp1b positive putative non-CM cells. Scale bar = 50µm. 5 images were analyzed in each group.

Figure S7



B

nrp1a

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ACCCGATACCTGCGGATCCGACCCATTAACTGGGAAACTGGCATCGCACTGCGGTTCGAGGTCTACGGATGCAAG - WT
CTGACCCGATACCTGCGGATCCGACCCATTAACTGGGAA----------CACTGCGGTTCGAGGTCTACGGATG
CTGACCCGATACCTGCGGATCCGACCCATTAACTGGGAAA------TCGCACTGCGGTTCGAGGTCTACGGATGC
CTGACCCGATACCTGCGGATCCGACCCATTAACTGGGAAACTG----------------------------
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nrp1b

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TCCTAGTAGCATACTAGCGCTAGTGACTGTAGCTGCTGTAGTCTCCATAGTAGTGGCAAGAGGAAGAGG   -WT
GTAGCATACTAGCGCTAGTGACTGTAGCTGCTGTAGTCTCCA-------GGCAAGAGGAAGAGGTGTTG
GTAGCATACTAGCGCTAGTGACTGTAGCTGCTGTAGTCT-------GTGGCAAGAGGAAGAGGTGTTGTG
GTAGCATACTAGCGCTAGTGACTGTAGCTGCTGTAGTCT----------GCAAGAGGAAGAGGTGTTGT
GTAGCATACTAGCGCTAGTGACTGTAGCTGCTGTAGTCT-------------------------------------
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Figure S7: CRISPR cas9 mediated genome editing in nrp1a and nrp1b. (A). T7E1 mutagenesis assay at the CRISPR target site in the nrp1a and nrp1b gene. The assay was performed on genomic DNA from 4 dpf embryos injected at the one-cell stage with Cas9 mRNA and either a gRNA against nrp1a or a gRNA against nrp1b. Cleavage bands (arrowheads) indicate the presence of mutations at the target site. (**B)**. Representative sequencing result showing the mutation sites.

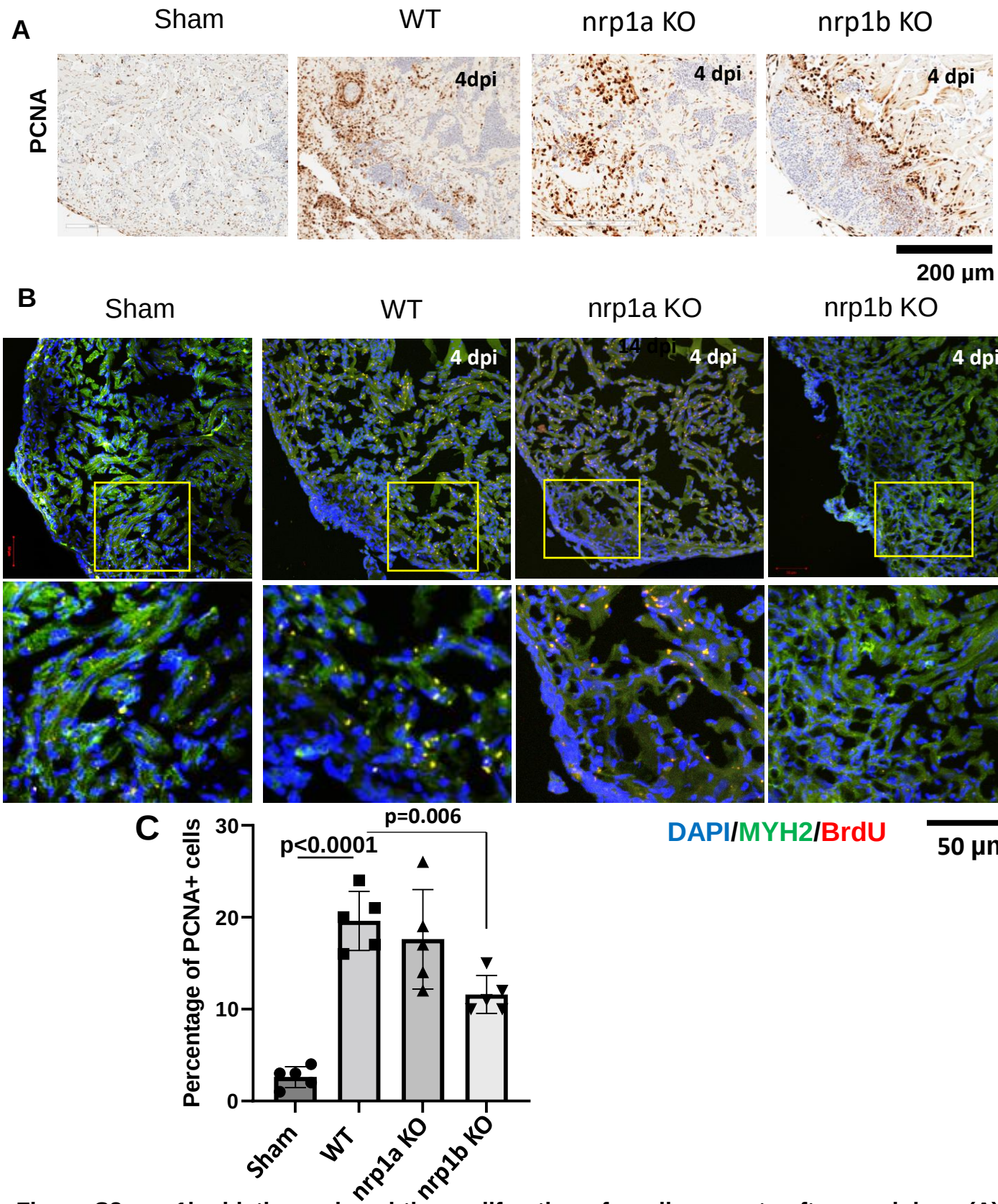

Figure S8: nrp1b ablation reduced the proliferation of cardiomyocyte after cryoinjury. (A). PCNA staining showing proliferative cells in WT-sham, WT- 4 dpi and CM specific nrp1a/nrp1b KO zebrafish adult heart section after 4 dpi. **(B)**. Representative confocal image showing the BrdU staining in WT- sham, WT-4 dpi and CM specific nrp1a/nrp1b KO zebrafish adult heart section after 4 dpi. **(C).** Quantification of the BrdU staining in B. Yellow square box indicates the analyzed area. Error bars represents the mean ± standard deviation (N=5 different images were analyzed for the quantification). Statistical significance between two groups was evaluated with Prism 9.0 software by using nonpaired, two-tailed Student's *t*-test. P values below 0.05 were considered significant and if lower as indicated.

Figure S9

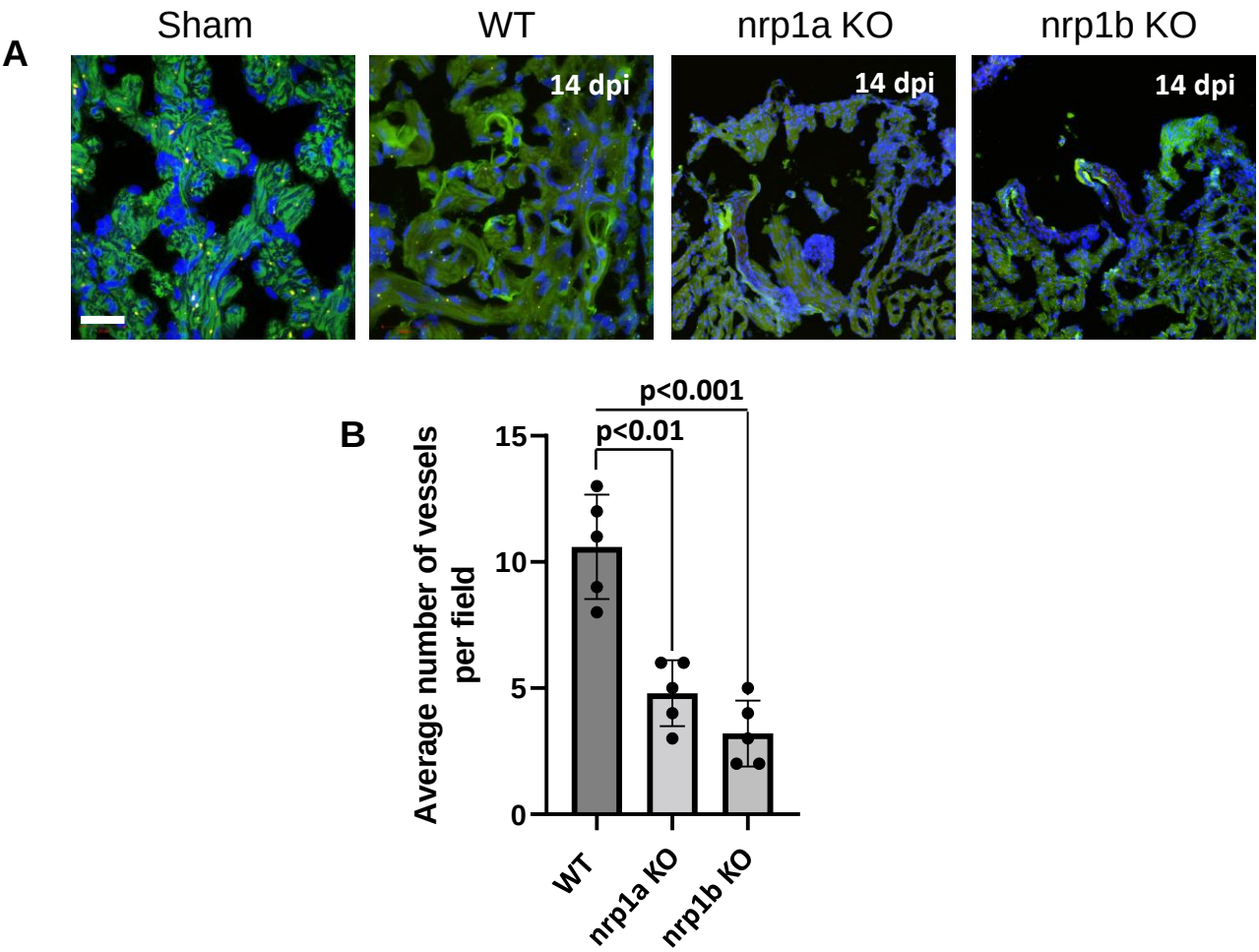

Figure S9: Neovascularization of the cryoinjured area is impaired in cardiomyocyte specific *nrp1a and nrp1b KO*. (A). Confocal image of ventricle section showing the *VE*-cadherin (green) in the WT-sham, WT- 4 dpi, and CM specific nrp1a/nrp1b KO zebrafish adult heart section (4 dpi). (**B)**. Quantification showing the number of neo-vessels in the four groups. Scale bar= 20µm. Error bars represents the mean ± SEM (N=5 images were analyzed per group). Statistical significance was evaluated with Prism 9.0 software by using *one* Way ANOVA. P values below 0.05 were considered significant and if lower as indicated.

Figure S10

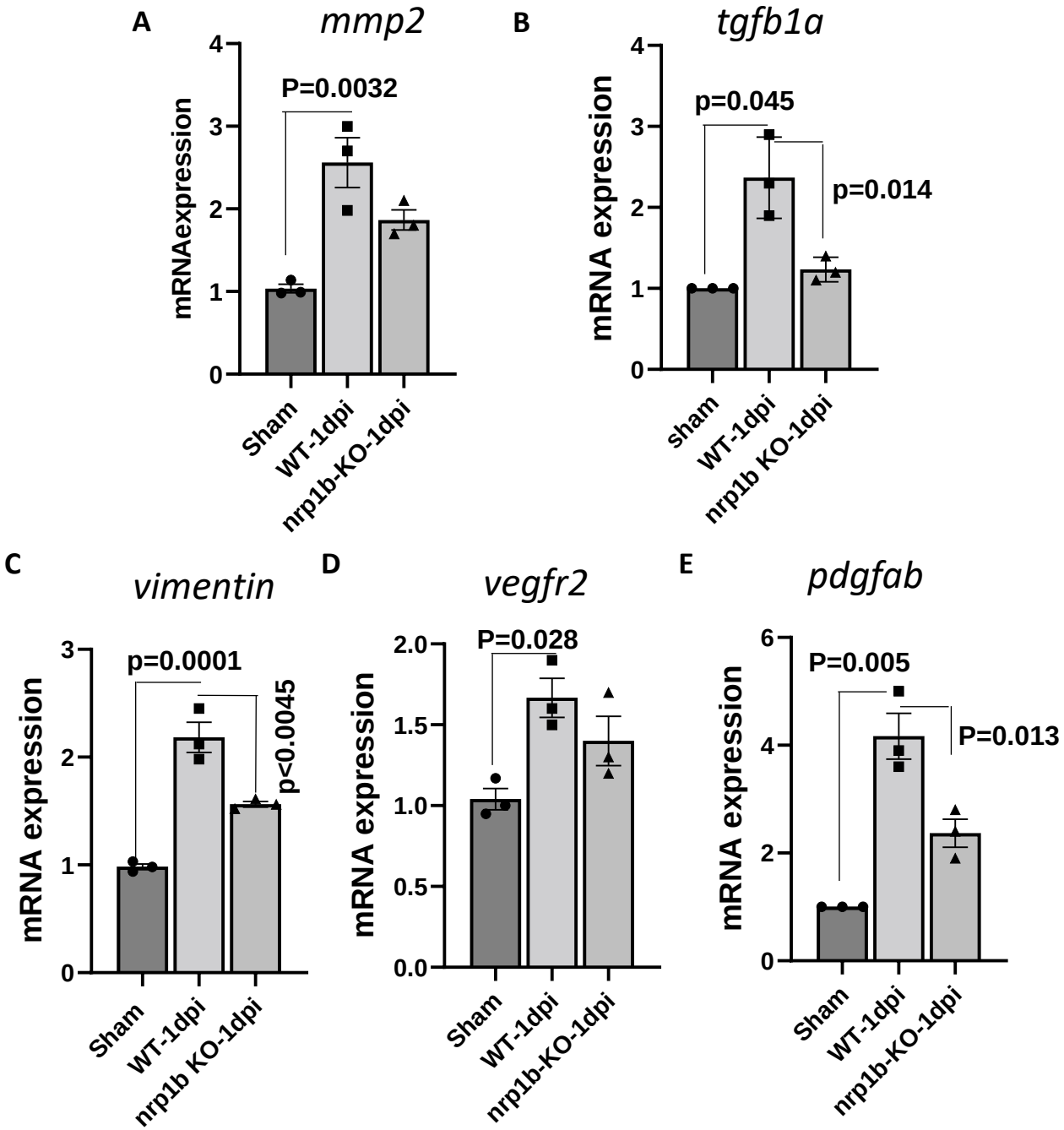

Figure S10: RNA expression of MMP2 and vimentin in the injured heart. (A) Relative mRNA expression of MMP2 in control, cardiac specific nrp1a and nrp1b KO heart after cryoinjury (1 dpi). (**B and C)** Relative mRNA expression of EMT marker genes expression in control, cardiac specific nrp1a and nrp1b KO heart. **(D and E)** Angiogenesis marker in. Error bars represents the mean ± SEM and the experiments were repeated at least three times.. Statistical significance was evaluated with Prism 9.0 software by using *one*-Way ANOVA with Sidak' s multiple comparison. P values below 0.05 were considered significant and if lower as indicated.

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Figure S11

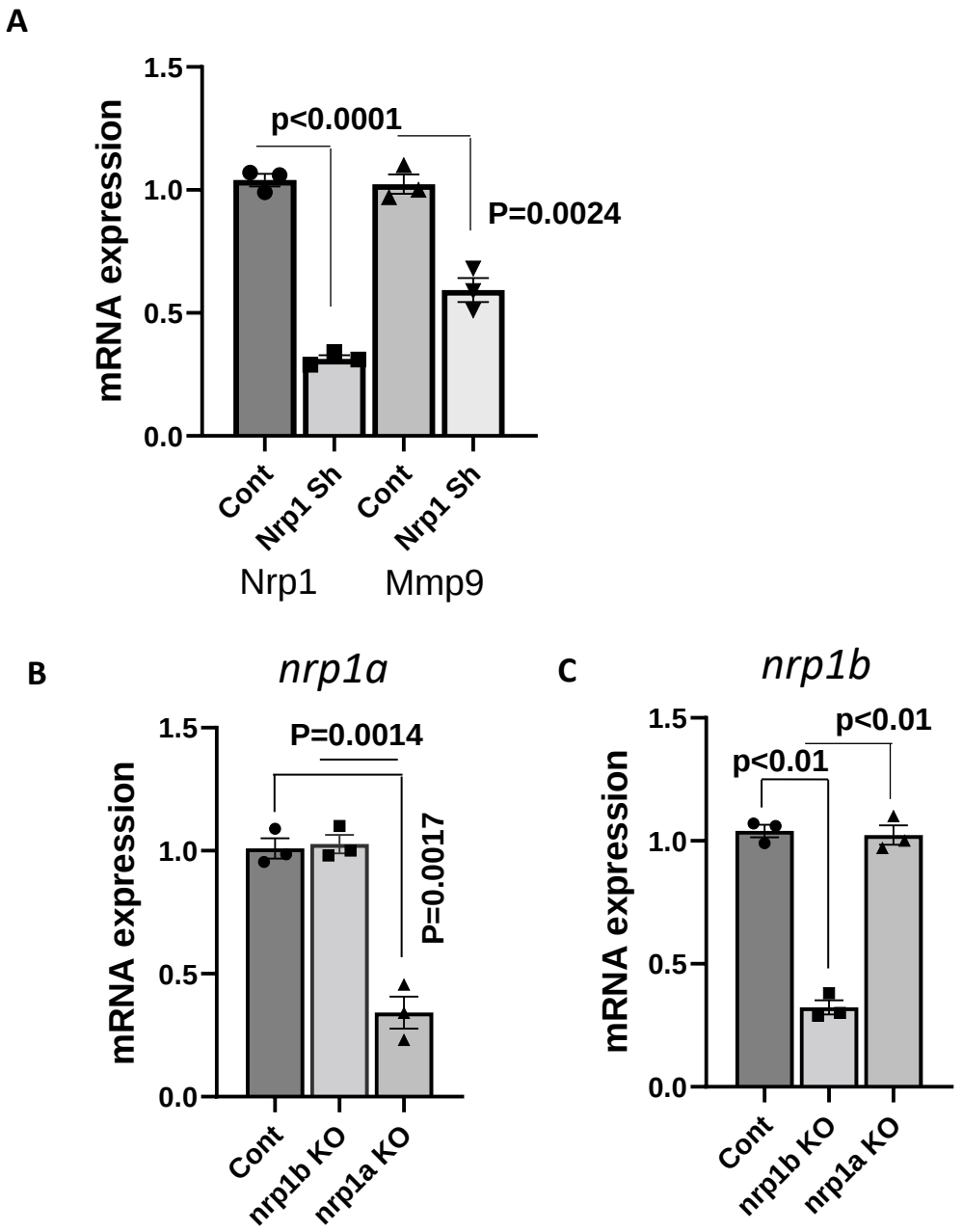

Figure S11: Compensatory effect *nrp1a and nrp1b*. (A) Relative mRNA expression of Nrp1 and MMP9 in control, and NRP1 shRNA#2 transfected mice ventricle cardiomyocyte (HL-1). **(B).** Relative mRNA expression of nrp1a in control, cardiac specific nrp1a and nrp1b KO heart. **(C)**. Relative mRNA expression of nrp1b in control, cardiac specific nrp1a and nrp1b KO heart. Error bars represents the mean ± SEM and the experiments were repeated at least three times. Statistical significance was evaluated with Prism 9.0 software by using Student's *t*-test in A and One way ANOVA in B and C. P values below 0.05 were considered significant and if lower as indicated.

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Supplemental Table 1. Primer sequences

Gene	Forwards	Reverse
Mmp9	TGATGTGCTTGGACCACGTAA	ACAGGAGCACCTTGCCTTTTC
Col1a2	AAGAACCCCGCTCGTACTTG	TCCAGTAGAAACCGCTGCTC
Mef2ca	CCGTCCATGAACATGAGCCT	ACCGGCTCCGACTTAATGTG
Mef2cb	GTACAACGAGCCACACGAGA	CACCTGCACTAGGTGGTCTG
Col1a1	GGCTTCCAGTTCGAGTATGG	ATGCAATGCTGTTCTTGCAG
Anp	GATGTACAAGCGCACACGTT	TCTGATGCCTCTTCTGTTGC
Vmhcl	TGTTGCAATCCAGACCGTCA	GCCACTTGTAGGGGTTGACA
Myh6	GCATTCATTTCGGGACGAGC	R GACGTGAAGCCAAGCACATC
Tnnt2a	AGCAGAGCAGCAGAGAATCC	R AGAGGTTTGCGTCGATCACC
Myh7	ATCAGGAGGTGGTTGTAGCC	GCAGGGTTAGCCTGGATGATT
nppa	GATGTACAAGCGCACACGTT	TCTGATGCCTCTTCTGTTGC
nppb	CATGGGTGTTTTAAAGTTTCTCC	CTTCAATATTTGCCGCCTTTAC
tnnt2c	GACCGAACGTGAGAAGAAGA	AGGACTTCCTGGTGGTTTTC
Actin	GCCTACTGGCCAGACGTCACAAATC	TCCAGCAAAACCGGCTTTGCACATAC
mouse MMP-9	CTTCTGGCGTGTGAGTTTCCA	ACTGCACGGTTGAAGCAAAGA
MMP13	CCTCCATATGAGGGCGTTGG	GATACATGAGTGCACCGGGA
pdgfab	CTGCTGCAACACCGGAAAC	GATCCTCTAACCGGACCAGC
tgfb1	CAACCGCTGGCTCTCATTTG	CCTCTCTGCTTGTCTAGCCC
tnfa	AGACACGACCACAGCACTTC	CGGCACATTGCCAAGAGTGT
MMP13	CCTCCATATGAGGGCGTTGG	GATACATGAGTGCACCGGGA

sgRNA sequence

nrp1a	1. AGAAATCCAAGATCAACCTGAGG 2. GGGAAACTGGCATCGCACTGCGG
nrp1b	1. GTCTCCATAGTAGTGGCAAGAGG 2. GTCTCACACTAACCTTGGTGGTAGG

Major Resources Table

In order to allow validation and replication of experiments, all essential research materials listed in the Methods should be included in the Major Resources Table below. Authors are encouraged to use public repositories for protocols, data, code, and other materials and provide persistent identifiers and/or links to repositories when available. Authors may add or delete rows as needed.

Animals (in vivo studies)

Species	Vendor or Source	Background Strain	Sex	Persistent ID / URL
Danio rerio	ZIRC	WT-AB (ZL1)	M/F	https://zebrafish.org/home/guide.php

Genetically Modified Animals

	Species	Vendor or Source	Background Strain	Other Information	Persistent ID / URL
Parent - Male	Danio rerio		CRISPR Cas9-nrp1a	Heat shock inducible	
Parent - Female	Danio rerio		CRISPR Cas9-nrp1b	Heat shock inducible	

Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot #	Persistent ID / URL
Neuropilin-1 (D62C6) Rabbit mAb	Cell Signaling	3725	1:1000		https://www.cellsignal.com/products/primary-antibodies/neuropilin-1-d62c6-rabbit-mab/3725?site-search-type=Products&N=4294956287&
MYH6	Abcam	ab207926	1:200		
BrdU (Bu20a) Mouse mAb	Cell Signaling	5292	1:200		https://www.cellsignal.com/products/primary-antibodies/brdu-bu20a-mouse-mab/5292?site-type=Products&N=4294956287&Ntt=brdu&fromPage=plp
VE-Cadherin (D87F2) XP® Rabbit mAb	Cell Signaling	2500	1:200		https://www.cellsignal.com/products/primary-antibodies/ve-cadherin-d87f2-xp-rabbit-mab/2 type=Products&N=4294956287&Ntt=d87f2&fromPage=plp
Anti-Actin antibody, Mouse monoclonal	Millipore Sigma	A3853	1:5000		https://www.sigmaaldrich.com/US/en/product/sigma/a3853
Anti-rabbit IgG, HRP-linked Antibody	Cell Signaling	7074S	1:10000	29	https://www.cellsignal.com/products/secondary-antibodies/anti-rabbit-igg-hrp-linked-antibo type=Products&N=4294956287&Ntt=7074s&fromPage=plp&_requestid=3345199
Anti-mouse IgG, HRP-linked Antibody #	Cell Signaling	7076	1:10000	35	https://www.cellsignal.com/products/secondary-antibodies/anti-mouse-igg-hrp-linked-antibo

DNA/cDNA Clones

Clone Name		Source / Repository	Persistent ID / URL
pDestTol2pA2-U6:gRNA		Addgene/doi: 10.1016/j.devcel.2015.01.032.	#63157/https://www.addgene.org/63157/

DOI [to be added]

pME-Cas9		Addgene/doi: 10.1016/j.devcel.2015.01.032.	#63154/ https://www.addgene.org/search/catalog/plasmids/?q=PME+
pENTR5'_ubi		Addgene/doi: 10.1016/j.devcel.2015.01.032.	https://www.addgene.org/27320/
P3E-PolyA		Tol2Kit	http://tol2kit.genetics.utah.edu/index.php/P3E-polyA

Cultured Cells

Name	Vendor or Source	Sex (F, M, or unknown)	Persistent ID / URL
HL-1 Cardiac Muscle Cell Line (SCC065)	Sigma		https://www.sigmaaldrich.com/US/en/product/mm/scc065

Data & Code Availability

Description	Source / Repository	Persistent ID / URL
NA	NA	NA

Other

Description	Source / Repository	Persistent ID / URL
NP-40 Lysis Buffer (BP-119)	Boston Bioproduct	https://www.bostonbioproducts.com/products/np-40-lysis-buffer-bp-119
Alexa Fluor™ 488 Phalloidin (A12379)	ThermoFisher Scientific	https://www.thermofisher.com/order/catalog/product/A12379
Claycomb Medium	51800C	
HyperScribe™ T7 High Yield Cy3 RNA Labeling Kit	K1061	https://www.apexbt.com/hyperscribetm-t7-high-yield-cy3-rna-labeling-kit.html
MS-550S transducer	VisualSonics	Vevo 3100
iWORX ECG instrument	SKU ZS-200	iWorx.Inc

ARRIVE GUIDELINES

The ARRIVE guidelines (https://arriveguidelines.org/) are a checklist of recommendations to improve the reporting of research involving animals. Key elements of the study design should be included below to better enable readers to scrutinize the research adequately, evaluate its methodological rigor, and reproduce the methods or findings.

Study Design

Groups	Sex	Age	Number (prior to experiment)	Number (after termination)	Littermates (Yes/No)	Other description

Sample Size: Please explain how the sample size was decided Please provide details of any a *prior* sample size calculation, if done.

Answer: The sample size was calculated based on the previous related study. **DOI: 10.1242/dev.174482**

Inclusion Criteria

Not done

DOI [to be added]

Exclusion Criteria

Not done

Randomization

Not done

Blinding

Not done

DOI [to be added]