nature portfolio

Peer Review File

FNDC4 alleviates cardiac ischemia/reperfusion injury through facilitating HIF1α-dependent cardiomyocyte survival and angiogenesis in male mice

Corresponding Author: Dr Can Hu

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript by Zhang et al investigates roles for FNDC4 in the hearts of mice subjected to cardiac ischemia/reperfusion. This is a very thorough and interesting study where the authors conclude that "...FNDC4 alleviates cardiac I/R injury through facilitating HIF1α-dependent cardiomyocyte survival and angiogenesis "

For the most part the experiment are well conceived, described and performed and generally interpreted reasonably. However, I have a few overarching and specific comments/questions for the authors, as follows:

Overarching Comments:

1- The authors' overall conclusion in the abstract did not describe the FNDC4 secretion components of their finding, or the FGF1 aspect of what they discovered.

2- The authors demonstrate that FNDC4 is secreted from cardiac myocytes, but never really show whether FNDC4 itself in secretion media of NRCMs has any protective effects on the NRCMs.

3- The authors didn't address whether intracellular FNDC4 might also have protective effects against I/R injury. Did the authors try to use NRCM conditioned media from Adh-FNDC4 expressing cells on other NRCMs subjected to simulated I/R? And how about trying the rFNDC4 in NRCMs?

4- The authors described the amount of rFNDC4 that was administered to mice and the route and frequency of administration, but never discussed whether the plasma levels of rFNDC4 ever reached therapeutic levels, i.e. the same dose as that which causes protection from simulated I/R damage of NRCMs?

Specific Comments:

5-p. 13 The authors stated that "....human FNDC4 protein was not detectable (N.D.) in the medium of AdCTRL-infected NRCMs, but increased in the medium of AdhFNDC4-infected NRCMs." And then a few sentences later they said ".....FNDC4 level in cell medium was also reduced by FNDC4 knockdown (Figure S4A-B)." This is confusing because if FNDC4 was not detected in AdV-Con NRCM media, then how was it shown that FNDC4 could be knocked down if it couldn't be detected in control media?

6-p. 16 The authors said that ".....FNDC4 overexpression in the myocardium mainly elevated HIF1α protein expression in I/R-stressed cardiomyocytes, but not in endothelial cells (Figure S11A)." But to me it looks like it goes up in both cell types on the immunoblot. Also, the quantitative analyses next to the blot are not labeled.

7-The title for Fig. S4 is " FNDC4 overexpression exacerbates sI/R-induced cardiomyocyte injury in vitro". However, as far as I can tell, the figure shows knockdown, not over expression.

Reviewer #2

(Remarks to the Author)

The manuscript by Zhang X et al. investigates the role of FNDC4 in cardiac damage in response to ischemia reperfusion model in mice. They have used FNDC4 overexpression in the heart and see a protective effect of FNDC4 against I/R heart damage. On the other hand deletion of FNDC4 from the cardiac muscle deteriorates the outcome of I/R. Finally, using rFNDC4 has a protective effect against cardiac damage, resulted from I/R. Overall there is new information about the FNDC4 biology, but none of the already known mechanisms with regards the FNDC4 signaling are checked or even discussed.

Major concerns are:
1. FNDC4 is not highly expressed in the heart. Please provide Ct values. Looking at the Western blots the signals look very clear. The antibody used against FNDC4 is a monoclonal antibody raised in mouse. It would be very important to see full blots in the supplementary sections, to judge the specificity of the antibody. Finally, only representative blots are shown, while the quantification od FNDC4 under different conditions is done based on quantification of blots that are not shown.
2. There is no information of the recombinant protein used? what part of the FNDC4 protein is cloned and produced as a recombinant protein ? is it produced in e.coli or mammalian cells or other ? is it endotoxin free or not ? is it commercially purchased or made in house ?
3. There are no measurements of body weight in response to AAVshFNDC4 or adFNDC4 or rFNDC4 interventions? Do the animals loose weight ?
4. AAV9 serotype is specific targeting the heart muscle? Please show at least qPCR of FNDC4 in other tissue such as liver and brain after AAV9ShFNDC4 deletion.
5. It is surprising that none of the published signaling mechanisms of FNDC4 have been explored or even mentioned in the discussion. For example, a. Is there a difference in macrophage numbers, activity and function in cardiac muscle in response to AAVshFNDC4, AdFNDC4 or rFNDC4 ?
6. What about Stat3? is this changing in the response to FNDC4 deletion or overexpression? Given that their published works on the role of STAT3 in heart muscle reparative response.
7. GPR116 was published to be a high affinity receptor. GPR116 is highly expressed in the heart and in endothelial cells, so it is indeed odd that GPR116 regulated signaling is not checked at all and not discussed at all.

Minor things:
8. Please first time you introduce an abbreviation in the text write the full name.

Reviewer #3

(Remarks to the Author)
In this manuscript, the role of FNDC4 for ischemia/reperfusion (I/R) injury was studied in mice, human samples and cell cultured. The authors report that FNDC4 level were increased 24h after I/R and that also hearts from patients with ischemia heart disease (IHD) exhibit higher FNDC4 level. Plasma level of FNDC4 were increased after I/R and in humans after acute myocardial infarction. High level of FNDC4 correlated with increased fraction of shortening and inversely with hscTnI. Higher plasma level of Fndc4 were associated with increased survival after MI. AAV9-mediated overexpression of FNDC4 resulted in lower tissue loss, apoptosis and hypertrophy as well as induction of angiogenic genes after I/R and shRNA-mediated knockdown had the opposite effect. Similar data were obtained in neonatal rat cardiac myocytes: FNDC4 overexpression was protective, knockdown deleterious. Overexpression of FNDC4 in HUVEC had no pro-angiogenic effect, indicative for a paracrine effect and subsequent experiments indicated that cardiac-myocyte-derived aFGF mediates the effect of FNDC4. In cardiac myocytes, FNDC4 increased stress-induced HIF1 protein level and inhibition of HIF1 prevented the protective effect of AAV-FNDC4. Mechanistically, the authors suggest that FNDC4 prevents degradation of HIF1 by the proteasome.
This is an interesting study, however, with a somewhat narrow focus. It is unclear why FNDC4 but not the other FNDCs mediate the effect and how FNDC4 inhibits the proteasome. This reviewer has the following specific comments:
1) Fig 1N: This is certainly not an ideal representation. How does the Kaplan-Meyer Curve look like? Are there differences significant?
2) Fig 5D/H: please also show mRNA expression of the VEGF isoforms.
3) Public available single cell data after MI should be explored to demonstrate the cell specific expression patterns and changes of all FNDCs after MI.
4) How is expression of FNDC4 controlled? Why does it increase after MI and I/R?
5) Is FNDC4 itself a HIF-dependent gene?
6) What makes FNDC4 unique? Paper lacks the bigger picture. How much can the different FNDCs replace each others?
7) Does modulation of FNDC4 alter the expression of the other FNDCs?
8) Fig 6E: What is the effect of PX-478 in the control-AAV group? To make the point of a specific function through FNDC4, the authors have to show that PX-478 has almost no effect under control condition (or shRNA against FNDC4).
9) Given that FNDC4 attenuated HIF degradation, it is unclear why it is not doing so under normoxia (Fig 6B). These data contradict the current concept, as during I/R HIF increases anyway due to attenuated degradation.
10) If FNDC4 indeed blocks proteasomal degradation, the level of hydroxylated and ubitinated HIF should increase in the cell. Please demonstrate this.
11) What is the effect of FNDC4 on proteasome activity? Please show.

12) What happens to other endogenous targets of the proteasome? Are they also increased?
13) How does FNDC4 inhibit the proteasome? Please identify the mechanism.
14) VEGF is an important HIF1-dependent gene. Why did VEGF level not increase in this study?

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
This manuscript by Zhang et al investigates roles for FNDC4 in the hearts of mice subjected to cardiac ischemia/reperfusion. This is a very thorough and interesting study where the authors conclude that "...FNDC4 alleviates cardiac I/R injury through facilitating HIF1α-dependent cardiomyocyte survival and angiogenesis "

For the most part the experiments are well conceived, described and performed, and generally interpreted well. I did not see any flaws in the data or their analyses. Moreover, the authors present sufficient data to demonstrate the viability of the mechanism that they conclude.

Reviewer #3

(Remarks to the Author)
With the revision, the manuscript has partially improved, but concerns remain. Numbers before the paragraphs refer to my previous points.
1) Fig 1N: Even if the authors do not have Kaplan Meyer-Curves, statistics can be applied to this figure and has to be performed.
2) Fig 5D: The p-value for VEGFa is 9.48^10-8. Why is that not significant?
3) scRNAseq after MI: This response is not acceptable. The information is important for the global relevance of the manuscript and has to be included.
4) This point is sufficiently addressed.
5) This point is sufficiently addressed.
6) This point is not sufficiently addressed at all. You are aiming on a journal with a broad audience and therefore you have to expand the focus of the study to increase the appeal. Your statement reads a bit like FNDC4 is like FNDC5. But why should a reader among all these FNDCs be interested specifically in FNDC4, when they are all do the same?
7) This point is sufficiently addressed.
8) Obviously, this response as expected, but it also imposes a big problem to the manuscript. Given that HIF is equally important under both conditions, the proposed effect is unlikely to be specific. In fact, it might not even exists, when the inhibitory effect of PX-478 in the control is identical to the AAV-group. This reviewer does not understand how the authors can exclude this problem? Or put it like this: What is the real evidence that the effect is mediated by HIF?
9) These authors only provide speculation but not a single experiment to address this crucial point. This is not acceptable.
10) This point is sufficiently addressed.
11) This point is sufficiently addressed.
12) With all respect, but have the authors considered that for example NFkappaB and NRF2 are also controlled by the proteasome? Such important transcription factors could also easily contribute to the mechanism suggested here. Such data are required.
13) This is an interesting observation, but the data are incomplete in that respect that they exclusively focus on the overexpression scenario. What happens to the components during I/R with and without overexpression and knockdown respectively? Is the downregulation also apparent on the protein level?
14) Okay, I can understand that this is not a central aspect of the manuscript.

Reviewer #4

(Remarks to the Author)
Thank you for your invitation.
The manuscript by Zhang et al. investigated the protective role of FNDC4 in heart under ischemia/reperfusion (I/R) in mice. And mechanically, FNDC4 promotes cardiomyocyte survival and angiogenesis through HIF1α dependent pathway.
The author's responses to most of the questions raised by Reviewer 2 were sufficient. But in the answer to to Question 3, the author should show the body weight data of mice receiving AAV9-hFNDC4, AAV9-shFndc4 or rFNDC4 treatment, which would answer the reviewer's question perfectly.
I believe that investigating the universal or specific receptors of FNDC4 in heart and how FNDC4 is secreted to extracellular space would help us better understand the role of FNDC4 in cardiac pathophysiology.

Version 2:

Reviewer comments:

Reviewer #3

(Remarks to the Author)
N/A

Reviewer #1 (Remarks to the Author):

This manuscript by Zhang et al investigates roles for FNDC4 in the hearts of mice subjected to cardiac ischemia/reperfusion. This is a very thorough and interesting study where the authors conclude that "...FNDC4 alleviates cardiac I/R injury through facilitating HIF1α-dependent cardiomyocyte survival and angiogenesis. " For the most part the experiment are well conceived, described and performed and generally interpreted reasonably. However, I have a few overarching and specific comments/questions for the authors, as follows:

Overarching Comments:

1-The authors' overall conclusion in the abstract did not describe the FNDC4 secretion components of their finding, or the FGF1 aspect of what they discovered.

Response: Thanks for your comment. According to the formatting instructions of the editors and the journals, the ABSTRACT should be changed to an unstructured form with 150 words or fewer. The FNDC4 secretion components of our finding as well as the FGF1 aspect of what we discovered were described in the RESULTS section of the ABSTRACT, so we did not further provide them in the CONCLUSION of the ABSTRACT due to the limited space and word limit.

2-The authors demonstrate that FNDC4 is secreted from cardiac myocytes, but never really show whether FNDC4 itself in secretion media of NRCMs has any protective effects on the NRCMs.

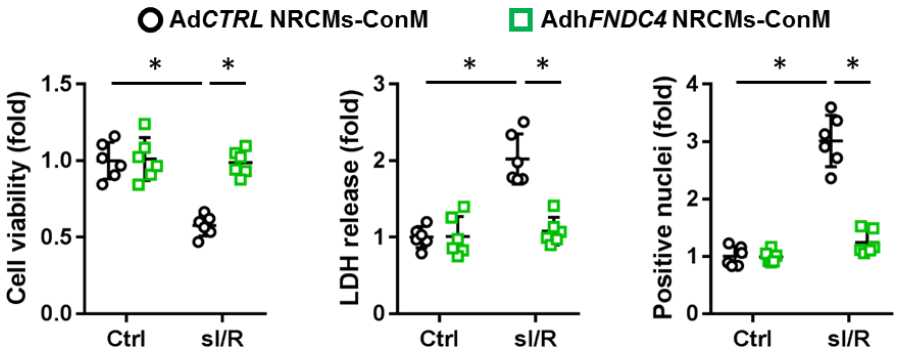
Response: Thanks for your comment. In our manuscript, we found that FNDC4 overexpression prevented, while FNDC4 knockdown exacerbated sI/R-induced cardiomyocyte injury in vitro (Figure 4 and Supplementary Figure 4). Further studies using commercial kits indicated that FNDC4 **in the cell medium** were elevated in FNDC4-overexpressed NRCMs, but decreased in FNDC4-silenced NRCMs (Figure 4B and Supplementary Figure 4B). In addition, we also prepared the rFNDC4 by cloning the **extracellular domain of FNDC4** as previously described[1, 2]. Next, we treated NRCMs with rFNDC4, and found that rFNDC4 dramatically prevented sI/R-induced cardiomyocyte injury and apoptosis in vitro **(the first paragraph and the last sentence of RESULTS 5**). Despite the lack of a commercial neutralizing antibody of FNDC4, the above findings could confirm that FNDC4 itself in secretion media of NRCMs provided protective effects on the NRCMs.

[1] Bosma M, Gerling M, Pasto J, et al. FNDC4 acts as an anti-inflammatory factor on macrophages and improves colitis in mice. Nat Commun. 2016;7:11314.

[2] Georgiadi A, Lopez-Salazar V, Merahbi RE, et al. Orphan GPR116 mediates the insulin sensitizing effects of the hepatokine FNDC4 in adipose tissue. Nat Commun. 2021;12(1):2999.

3-The authors didn't address whether intracellular FNDC4 might also have protective effects against I/R injury. Did the authors try to use NRCM conditioned media from Adh-FNDC4 expressing cells on other NRCMs subjected to simulated I/R? And how about trying the rFNDC4 in NRCMs?

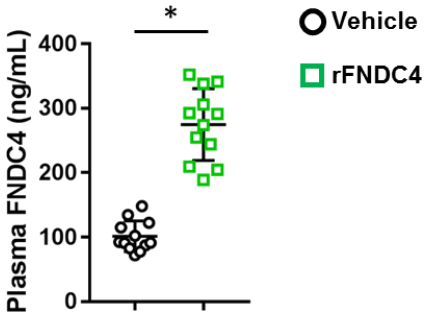
Response: Thanks for your comment. As indicated in the response to the last comment, the protective role of rFNDC4 in NRCMs has been determined in the study, and our findings revealed that rFNDC4 treatment dramatically prevented sI/R-induced cardiomyocyte injury and apoptosis in vitro **(the first paragraph and the last sentence of RESULTS 5**). According to the suggestions by the reviewer, we also treated sI/R-stimulated NRCMs with the conditioned medium from Adh*FNDC4*- or Ad*CTRL*-infected NRCMs. As expected, the conditioned medium from Adh*FNDC4*-infected NRCMs could prevent sI/R-induced cardiomyocyte injury in vitro (data were shown as following). The above findings indicated that FNDC4 in secretion media of NRCMs provided protective effects on the NRCMs. However, no current data were available to support whether intracellular FNDC4 might also have protective effects against I/R injury. We have discussed and added this limitation in our revised DISCUSSION.



4-The authors described the amount of rFNDC4 that was administered to mice and the route and frequency of administration, but never discussed whether the plasma levels of rFNDC4 ever reached therapeutic levels, i.e. the same dose as that which causes protection from simulated I/R damage of NRCMs?

Response: Thanks for your insightful comment. According to the findings by Georgiadi et al., this amount, route and frequency of rFNDC4 administration was sufficient to restore the plasma FNDC4 level in high fat

diet-treated mice, and prevent obesity-related pre-diabetes in mice[1]. In our study, we also treated mice with this dose of rFNDC4, and our preliminary experiments showed that rFNDC4 could elevate the plasma level of FNDC4 by 2.71 fold. To address this comment, we provided the preliminary data as following. As for the comparison of rFNDC4 in mice and NRCMs, we did not done this because we would investigate the optimal dose and route of FNDC4 administration to achieve a better therapeutic outcome in our further study with the primates. In addition, we would also improve the structure and stability of rFNDC4 to make it more suitable for therapeutic use.



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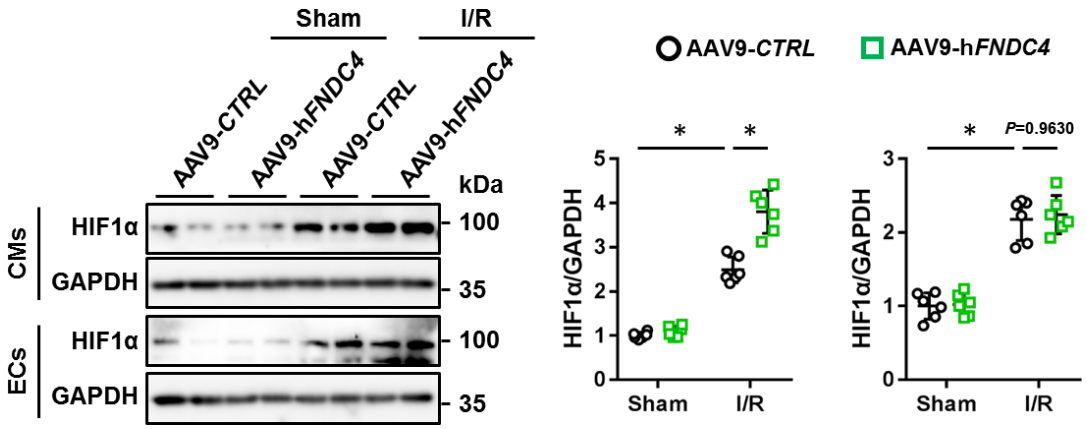
Response: Thanks for your comment. “As shown in Figure 4B, human FNDC4 protein was not detectable (N.D.) in the medium of Ad*CTRL*-infected NRCMs, but increased in the medium of Adh*FNDC4*-infected NRCMs.” In this experiments, **rat cardiomyocytes** were overexpressed with **human FNDC4**, and then, **human FNDC4** expression in the medium was measured using a commercial **human FNDC4 ELISA Kit** (#MBS9332722), which could not identify rat FNDC4 in the medium of Ad*CTRL*-infected NRCMs. So, we said that **human FNDC4** protein was not detectable (N.D.) in the medium of Ad*CTRL*-infected NRCMs. However, **human FNDC4** could be detected in the medium of rat cardiomyocytes overexpressing human FNDC4 (Adh*FNDC4*-infected NRCMs). In Supplementary Figure 4A-B, FNDC4 in the medium of **rat cardiomyocytes** were measured using a **rat FNDC4 ELISA Kit** (#MBS9399687), so FNDC4 could be detected both at baseline and after FNDC4 knockdown.

6-p. 16 The authors said that ". FNDC4 overexpression in the myocardium mainly elevated HIF1α protein expression in I/R-stressed cardiomyocytes, but not in endothelial cells (Figure S11A)." But to me it looks like it goes up in both cell types on the immunoblot. Also, the quantitative analyses next to the blot are not labeled.

Response: Thanks for your comment. The blot was one of the replicates of the experiments, and all blots were used for generating the quantitative results. As shown below, we added the quantitative results next to the blots. P was 0.9630, and represented no significance.



7-The title for Fig. S4 is " FNDC4 overexpression exacerbates sI/R-induced cardiomyocyte injury in vitro". However, as far as I can tell, the figure shows knockdown, not over expression.

Response: Thanks for your comment. As suggested, we revised the description.

Reviewer #2 (Remarks to the Author):

The manuscript by Zhang X et al. investigates the role of FNDC4 in cardiac damage in response to ischemia reperfusion model in mice. They have used FNDC4 overexpression in the heart and see a protective effect of FNDC4 against I/R heart damage. On the other hand deletion of FNDC4 from the cardiac muscle deteriorates the outcome of I/R. Finally, using rFNDC4 has a protective effect against cardiac damage, resulted from I/R. Overall there is new information about the FNDC4 biology, but none of the already known mechanisms with regards the FNDC4 signaling are checked or even discussed.

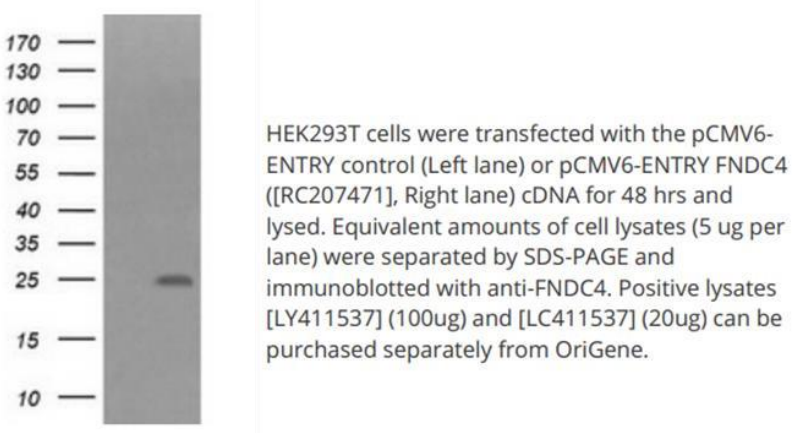
Major concerns are:

1. FNDC4 is not highly expressed in the heart. Please provide Ct values. Looking at the Western blots the signals look very clear. The antibody used against FNDC4 is a monoclonal antibody raised in mouse. It would be very important to see full blots in the supplementary sections, to judge the specificity of the antibody. Finally, only representative blots are shown, while the quantification od FNDC4 under different conditions is done based on quantification of blots that are not shown.

Response: Thanks for your comment. The Ct values of FNDC4 mRNA in mouse hearts were provided as following. From our PCR and western blot data, we found that FNDC4 was moderately expressed in mouse hearts. The anti-FNDC4 antibody (Catalog No. CF505459) was purchased from OriGene, and the specificity of this antibody was validated by western blot in the official website https://www.origene.com/catalog/antibodies/primary-antibodies/cf505459/fndc4-mouse-monoclonal-antibody-clone-id-oti3e10. The western blot image was extracted from the website and pasted as following. As suggested, we also provided the full blots in the supplementary sections. The representative blots were shown to make the quantification look more intuitive.

Sample	Rep	Targets	References	Mean Cp	Mean Cp	Target/Ref
Sample-1	Rep-1	*Fndc4*	*Gapdh*	23.680371	14.455349	1.67E-03
	Rep-2	*Fndc4*	*Gapdh*	23.719352	14.343492	1.51E-03
	Rep-3	*Fndc4*	*Gapdh*	23.602347	14.417336	1.72E-03
Sample-2	Rep-1	*Fndc4*	*Gapdh*	23.934194	13.602224	7.76E-04
	Rep-2	*Fndc4*	*Gapdh*	23.978454	13.653385	7.80E-04
	Rep-3	*Fndc4*	*Gapdh*	23.912406	13.635722	8.06E-04
Sample-3	Rep-1	*Fndc4*	*Gapdh*	23.511709	14.430549	1.85E-03
	Rep-2	*Fndc4*	*Gapdh*	23.568482	14.438772	1.79E-03
	Rep-3	*Fndc4*	*Gapdh*	23.546273	14.485449	1.87E-03
Sample-4	Rep-1	*Fndc4*	*Gapdh*	24.639919	14.330101	7.88E-04
	Rep-2	*Fndc4*	*Gapdh*	24.651867	14.440013	8.43E-04
	Rep-3	*Fndc4*	*Gapdh*	24.53693	14.378918	8.75E-04
Sample-5	Rep-1	*Fndc4*	*Gapdh*	23.662097	13.934328	1.18E-03
	Rep-2	*Fndc4*	*Gapdh*	23.961457	14.158789	1.12E-03
	Rep-3	*Fndc4*	*Gapdh*	24.025567	14.204273	1.11E-03
Sample-6	Rep-1	*Fndc4*	*Gapdh*	24.436995	14.595934	1.09E-03
	Rep-2	*Fndc4*	*Gapdh*	24.464848	14.655292	1.11E-03
	Rep-3	*Fndc4*	*Gapdh*	24.600068	14.59476	9.73E-04

The Ct values of FNDC4 in mouse hearts



The western blot image was extracted from the website

2. There is no information of the recombinant protein used? what part of the FNDC4 protein is cloned and produced as a recombinant protein ? is it produced in e.coli or mammalian cells or other ? is it endotoxin free or not ? is it commercially purchased or made in house ?

Response: Thanks for your comment. The recombinant FNDC4 was prepared as previously described, and we cited the references in our revised manuscript [1, 2]. The extracellular fragment of FNDC4 was cloned, and then produced in mammalian cells and as such being free of endotoxin. The information was provided in "Methods- Animal studies", and labelled with RED.

[1] Bosma M, Gerling M, Pasto J, et al. FNDC4 acts as an anti-inflammatory factor on macrophages and improves colitis in mice. Nat Commun. 2016;7:11314.

[2] Georgiadi A, Lopez-Salazar V, Merahbi RE, et al. Orphan GPR116 mediates the insulin sensitizing effects of the hepatokine FNDC4 in adipose tissue. Nat Commun. 2021;12(1):2999.

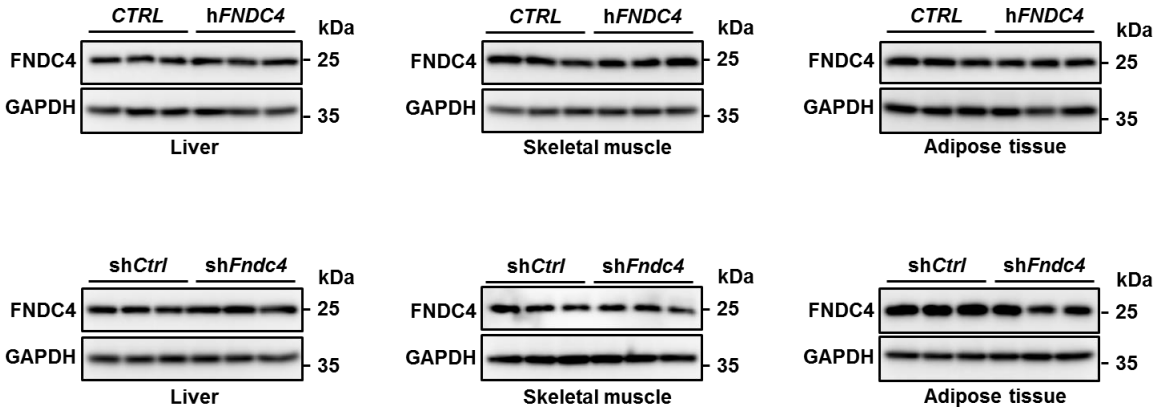
3. There are no measurements of body weight in response to AAVshFNDC4 or adFNDC4 or rFNDC4 interventions? Do the animals loose weight ?

Response: Thanks for your comment. As indicated by the authors, we re-evaluated the body weight of mice receiving AAV9-h*FNDC4*, AAV9-sh*Fndc4* or rFNDC4 treatment, and found that either FNDC4 overexpression/supplementation or FNDC4 knockdown made no differences in body weight. The results were also consistent with a previous study by Georgiadi et al[1].

[1] Georgiadi A, Lopez-Salazar V, Merahbi RE, et al. Orphan GPR116 mediates the insulin sensitizing effects of the hepatokine FNDC4 in adipose tissue. Nat Commun. 2021;12(1):2999.

4. AAV9 serotype is specific targeting the heart muscle? Please show at least qPCR of FNDC4 in other tissue such as liver and brain after AAV9ShFNDC4 deletion.

Response: Thanks for your comment. As indicated in the manuscript "Methods-Reagents", AAV9 is a cardiotropic viral vector, which mainly targets the myocardium. To enhance the tissue- and cell-specific impacts, we also used a cardiomyocyte-specific cTnT promoter to induce FNDC4 overexpression or knockdown in the cardiomyocytes. In our unpublished data of a previous study, we also determined FNDC4 expression in different tissues with or without FNDC4 overexpression or knockdown, and the results were provided as following. From the theoretical evidence and experimental data, we found that FNDC4 was only overexpressed or knocked down in the myocardium.



5. It is surprising that none of the published signaling mechanisms of FNDC4 have been explored or even mentioned in the discussion. For example, a. Is there a difference in macrophage numbers, activity and function in cardiac muscle in response to AAVshFNDC4, AdFNDC4 or rFNDC4 ?

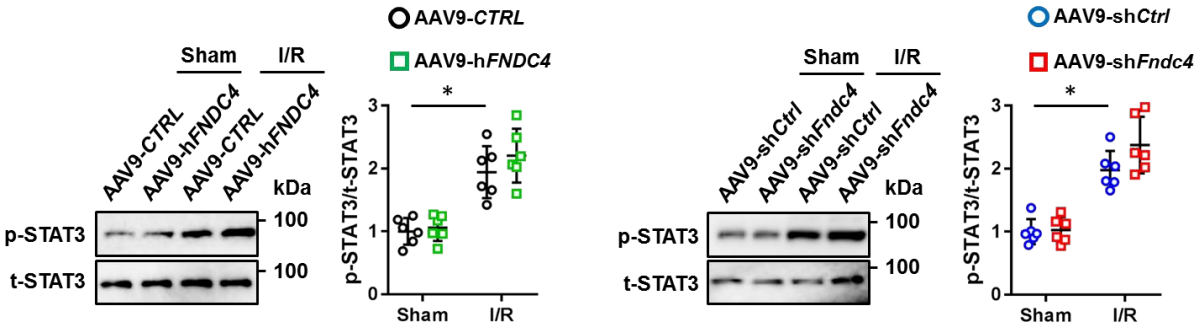
Response: Thanks for your comment. In our study, we found that cardiac-specific FNDC4 overexpression prevented, while cardiac-specific FNDC4 knockdown aggravated cardiac I/R injury. Using unbiased transcriptome analysis, we found that FNDC4 blocked the proteasomal degradation of HIF1α and subsequently activated HIF1α signaling pathway to exert the cardioprotective effects. Meanwhile, FNDC4 did not directly stimulate angiogenesis of endothelial cells, but increased the expression and secretion of FGF1 from cardiomyocytes to enhance angiogenesis in a paracrine manner. **The signaling mechanisms of FNDC4 in cardiac I/R injury were screened by the unbiased transcriptome analysis. The unbiased transcriptome analysis revealed that the published signaling mechanisms were not significantly altered**

by FNDC4 under cardiac I/R stress, so we did not explore these mechanisms. As suggested, we discussed these published mechanisms in the revised manuscript.

6. What about Stat3? is this changing in the response to FNDC4 deletion or overexpression? Given that their published works on the role of STAT3 in heart muscle reparative response.

Response: Thanks for your comment. Our unbiased transcriptome analysis revealed that JAK-STAT signaling pathway was unaltered by FNDC4 overexpression during cardiac I/R injury. Consistently, we also determined that FNDC4 overexpression or knockdown did not affect STAT3 activation. The results of unbiased transcriptome analysis and western blots were provided as following:

id	Term	Classific	Classific	ListHits	ListTotal	PopHits	PopTotal	p-value	q-value	Enrichmer	geneID	hyperlink_
mmu04630	JAK-STAT signaling pathway	Environme	Signal tr	11	483	171	8754	0.343828	1	1.165886	Cdkn1a;Cs	mmu04630



7. GPR116 was published to be a high affinity receptor. GPR116 is highly expressed in the heart and in endothelial cells, so it is indeed odd that GPR116 regulated signaling is not checked at all and not discussed at all.

Response: Thanks for your insightful comment. As indicated by the reviewer and a previous study, GPR116 is highly expressed in the heart and in endothelial cells, which plays critical roles in angiogenesis[1]. Meanwhile, Georgiadi et al. previously identified GPR116 as a receptor of FNDC4 in the white adipose tissue[2]. So, it is reasonable to investigate whether GPR116 in endothelial cells mediated the angiogenic effect of FNDC4 during cardiac I/R injury. However, our study revealed that endothelial cells were neither the origin nor the direct target of FNDC4. Moreover, Niaudet et al. found that GPR116 knockout dramatically increased vascular density, which was contrary to our findings showing that FNDC4 facilitated angiogenesis in I/R-stressed hearts[3]. Based on these studies, we did not explore the involvement of GPR116 in FNDC4-

mediated cardioprotection against I/R injury. We have added these in our revised DISCUSSION.

[1] Wallgard E, Larsson E, He L, et al. Identification of a core set of 58 gene transcripts with broad and specific expression in the microvasculature. Arterioscler Thromb Vasc Biol. 2008;28(8):1469-76.

[2] Georgiadi A, Lopez-Salazar V, Merahbi RE, et al. Orphan GPR116 mediates the insulin sensitizing effects of the hepatokine FNDC4 in adipose tissue. Nat Commun. 2021;12(1):2999.

[3] Niaudet C, Petkova M, Jung B, et al. Adgrf5 contributes to patterning of the endothelial deep layer in retina. Angiogenesis. 2019;22(4):491-505.

Minor things:

8. Please first time you introduce an abbreviation in the text write the full name.

Response: Thanks for your comment. We introduced the full name of the abbreviations in the text as suggested.

Reviewer #3 (Remarks to the Author):

In this manuscript, the role of FNDC4 for ischemia/reperfusion (I/R) injury was studied in mice, human samples and cell cultured. The authors report that FNDC4 level were increased 24h after I/R and that also hearts from patients with ischemia heart disease (IHD) exhibit higher FNDC4 level. Plasma level of FNDC4 were increased after I/R and in humans after acute myocardial infarction. High level of FNDC4 correlated with increased fraction of shortening and inversely with hscTnI. Higher plasma level of Fndc4 were associated with increased survival after MI. AAV9-mediated overexpression of FNDC4 resulted in lower tissue loss, apoptosis and hypertrophy as well as induction of angiogenic genes after I/R and shRNA-mediated knockdown had the opposite effect. Similar data were obtained in neonatal rat cardiac myocytes: FNDC4 overexpression was protective, knockdown deleterious. Overexpression of FNDC4 in HUVEC had no pro-angiogenic effect, indicative for a paracrine effect and subsequent experiments indicated that cardiac-myocyte-derived aFGF mediates the effect of FNDC4. In cardiac myocytes, FNDC4 increased stress-induced HIF1 protein level and inhibition of HIF1 prevented the protective effect of AAV-FNDC4. Mechanistically, the authors suggest that FNDC4 prevents degradation of HIF1 by the proteasome. This is an interesting study, however, with a somewhat narrow focus. It is unclear why FNDC4 but not the other FNDCs mediate the

effect and how FNDC4 inhibits the proteasome. This reviewer has the following specific comments:

1) Fig 1N: This is certainly not an ideal representation. How does the Kaplan-Meyer Curve look like? Are there differences significant?

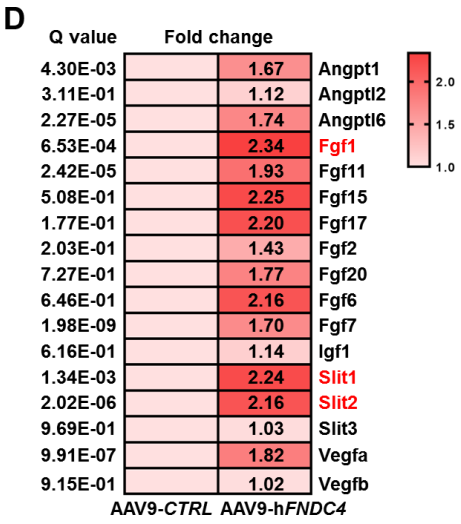
Response: Thanks for your insightful comment. We really agreed with your opinion that Fig 1N was certainly not an ideal representation, and that the Kaplan-Meyer Curve would be better for the follow-up. Actually, only a small amount of patients were included for analysis, and we did not obtain the survival status of these patients at different times. As suggested by the reviewer, we would expand the sample numbers and obtain the survival status of these patients at different times to calculate a Kaplan-Meyer Curve. We felt really sorry for lacking the data.

2) Fig 5D/H: please also show mRNA expression of the VEGF isoforms.

Response: Thanks for your comment. The unbiased transcriptome data revealed that the mRNA levels of Vegfa, Vegfb and Vegfd were unaffected by FNDC4 overexpression in I/R-stressed hearts (data were shown as following). As suggested by the reviewer, we provided the mRNA expressions of Vegfa and Vegfb in revised Fig 5D, as they were positively regulated by FNDC4 overexpression.

gene_id	BaseMean_	BaseMean_	BaseMean_	FoldChang	log2FoldC	p-value	q-value
Vegfa	19294.14	13700.44	24887.85	1.816556	0.861206	9.48E-08	9.91E-07
Vegfb	2242.944	2222.899	2262.99	1.018173	0.025983	0.869126	9.15E-01
Vegfd	532.5592	548.5405	516.5778	0.941977	-0.08624	0.611665	0.725247

The raw transcriptome data of Vegf isoforms



Revised Fig 5D

3) Public available single cell data after MI should be explored to demonstrate the cell specific expression patterns and changes of all FNDCs after MI.

Response: Thanks for your insightful comment. We had difficulty to obtain and analyze the public available single cell data after MI to demonstrate the cell-specific expression patterns and changes of all FNDCs. Actually, various FNDCs have already been implicated in the pathogenesis of cardiovascular diseases, including FNDC1, FNDC3 and FNDC5 (described in the INTRODUCTION). FNDC2 is not expressed in mouse tissues. Our previous studies also showed that FNDC5 overexpression dramatically prevented doxorubicin- and aging-induced cardiac injury and dysfunction[1, 2]. FNDC4 shows the strongest homology with FNDC5; however, its role in cardiovascular diseases remain elusive. Of note, our unpublished study determined that cardiac-specific overexpression of FNDC4 could prevent cardiac aging in mice (**revised by the journal Cardiovascular Research**). Based on these findings, we thus investigated the role and mechanisms of FNDC4 in cardiac I/R injury.

[1] Zhang X, Hu C, Kong CY, et al. FNDC5 alleviates oxidative stress and cardiomyocyte apoptosis in doxorubicin-induced cardiotoxicity via activating AKT. Cell Death Differ. 2020;27(2):540-555.

[2] Hu C, Zhang X, Hu M, et al. Fibronectin type III domain-containing 5 improves aging-related cardiac dysfunction in mice. Aging Cell. 2022;21(3):e13556.

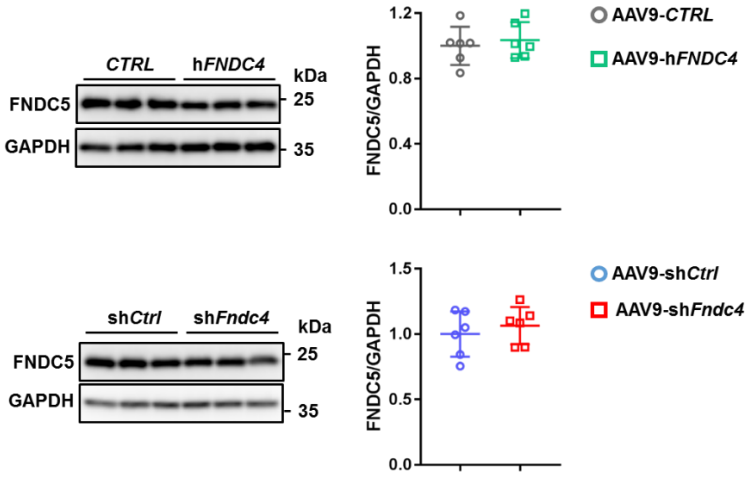
4) How is expression of FNDC4 controlled? Why does it increase after MI and I/R?

5) Is FNDC4 itself a HIF-dependent gene?

Response: Thank you for these two insightful comments. As suggested by the reviewer, we explored whether FNDC4 expression was controlled by HIF1α. We identified various putative binding sites of HIF1α (5′-RCGTG-3′) in the promoter of human, mouse or rat FNDC4. Previous studies have shown that HIF1α expression in the heart was elevated by I/R stimulation, and our present study also detected a significant increase of FNDC4 expression during cardiac I/R injury in vivo and in vitro. Based on these, we speculated that FNDC4 upregulation in response to cardiac I/R injury was dependent on HIF1α. To address this comment, we treated I/R-stressed mice or sI/R-stimulated NRCMs with PX-478 to inhibit HIF1α. As shown in revised Fig 1, HIF1α inhibition dramatically suppressed FNDC4 upregulation during cardiac I/R injury in vivo and in vitro. These results suggest that FNDC4 and HIF1α create a positive feedback to prevent I/R-induced cardiac injury and dysfunction.

6) What makes FNDC4 unique? Paper lacks the bigger picture. How much can the different FNDCs replace each others?

Response: Thanks for your comment. **As indicated in the response to the third comment**, various FNDCs have already been implicated in the pathogenesis of cardiovascular diseases, including FNDC1, FNDC3 and FNDC5 (described in the INTRODUCTION). FNDC2 is not expressed in mouse tissues. Our previous studies also showed that FNDC5 overexpression dramatically prevented doxorubicin- and aging-induced cardiac injury and dysfunction[1, 2]. **FNDC4 shows the strongest homology with FNDC5**; however, its role in cardiovascular diseases remain elusive. Of note, **our unpublished study determined that cardiac-specific overexpression of FNDC4 could prevent cardiac aging in mice (revised by the journal Cardiovascular Research)**. Based on these findings, we thus investigated the role and mechanisms of FNDC4 in cardiac I/R injury. Despite the strongest homology of FNDC4 and FNDC5, we found that FNDC4 knockdown or overexpression did not affect FNDC5 expression (unpublished data about the role of FNDC4 in cardiac aging), thereby excluding the potential involvement of FNDC5. The unpublished data were provided as following.



[1] Zhang X, Hu C, Kong CY, et al. FNDC5 alleviates oxidative stress and cardiomyocyte apoptosis in doxorubicin-induced cardiotoxicity via activating AKT. Cell Death Differ. 2020;27(2):540-555.

[2] Hu C, Zhang X, Hu M, et al. Fibronectin type III domain-containing 5 improves aging-related cardiac dysfunction in mice. Aging Cell. 2022;21(3):e13556.

7) Does modulation of FNDC4 alter the expression of the other FNDCs?

Response: Thanks for your comment. **As indicated in the response to the 6th comment**, FNDC4 knockdown

or overexpression did not affect FNDC5 expression, thereby excluding the potential involvement of FNDC5. As suggested by the reviewer, we re-analyzed the unbiased transcriptome data, and found that FNDC4 overexpression-mediated cardioprotection was irrelevant with the upregulation of other FNDCs, including FNDC3a, FNDC3b, FNDC5, FNDC7 and FNDC8. The unbiased transcriptome data were pasted as following.

gene_id	BaseMean_	BaseMean_	BaseMean_	FoldChang	log2FoldC	p-value	q-value
Fndc3a	3644.352	4064.681	3224.023	0.793109	-0.33441	0.032612	0.079332
Fndc3b	4790.067	4034.766	5545.369	1.374298	0.458695	0.002147	0.007765
Fndc5	3.991706	7.047698	0.935714	0.133377	-2.90642	0.055378	0.122351
Fndc7	85.92318	92.24004	79.60632	0.863217	-0.21221	0.483332	0.617536
Fndc8	42.82381	39.04606	46.60157	1.195346	0.257428	0.64259	0.750334

8) Fig 6E: What is the effect of PX-478 in the control-AAV group? To make the point of a specific function through FNDC4, the authors have to show that PX-478 has almost no effect under control condition (or shRNA against FNDC4).

Response: Thanks for your comment. The cardioprotective role of HIF1α has been validated by previous studies, and inhibiting HIF1α dramatically aggravated cardiac I/R injury[1, 2]. Therefore, PX-478 treatment in the AAV9-*CTRL* group could definitely exacerbate I/R-induced cardiac injury and dysfunction. Our study determined that FNDC4 overexpression prevented cardiac I/R injury through activating HIF1α, and HIF1α inhibition dramatically abolished the cardioprotection of FNDC4 in vivo and in vitro. Therefore, we showed that PX-478 treatment reversed the protective effects of FNDC4 against cardiac I/R injury (Figure 6 and Supplementary Figure 11-12).

[1] Liu X, Zhou L, Cheng J, et al. SENP1 protects against myocardial ischaemia/reperfusion injury via a HIF1alpha-dependent pathway. Cardiovasc Res. 2014;104(1):83-92.

[2] Du M, Huang K, Huang D, et al. Renalase is a novel target gene of hypoxia-inducible factor-1 in protection against cardiac ischaemia-reperfusion injury. Cardiovasc Res. 2015;105(2):182-91.

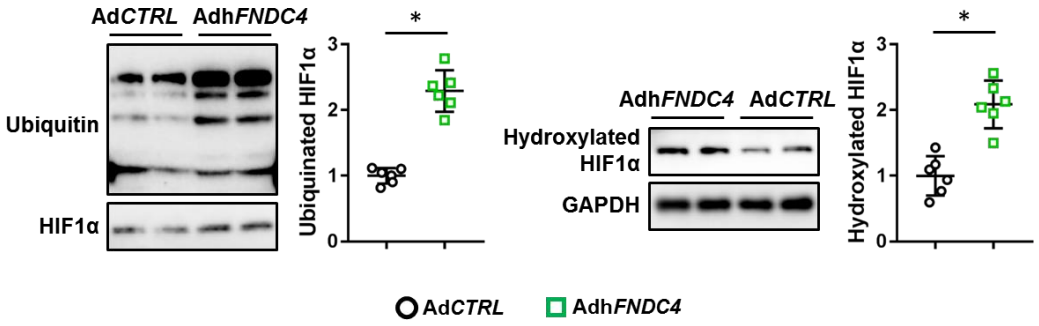
9) Given that FNDC4 attenuated HIF degradation, it is unclear why it is not doing so under normoxia (Fig 6B). These data contradict the current concept, as during I/R HIF increases anyway due to attenuated degradation.

Response: Thanks for your comment. We had no data to address this comment, but tried to cite some references for explanation. Protein homeostasis is modulated by various factors, including transcription, post-transcriptional modification, translation and post-translational modification (ubiquitination, acetylation,

Neddylation, SUMOylation, etc). Ubiquitination and proteasomal degradation represent one of the mechanisms of post-translational modification. Under normoxia, other regulatory mechanisms are complete and may compensate for FNDC4-mediated regulation on HIF1α expression. So, the expression of HIF1α is unaffected by FNDC4 overexpression, despite the inhibition of proteasomal degradation of HIF1α. Under I/R stimulation, these regulatory mechanisms might be directly modulated by ischemic stimuli, and subsequently cannot compensate for FNDC4-mediated regulation on HIF1α expression.

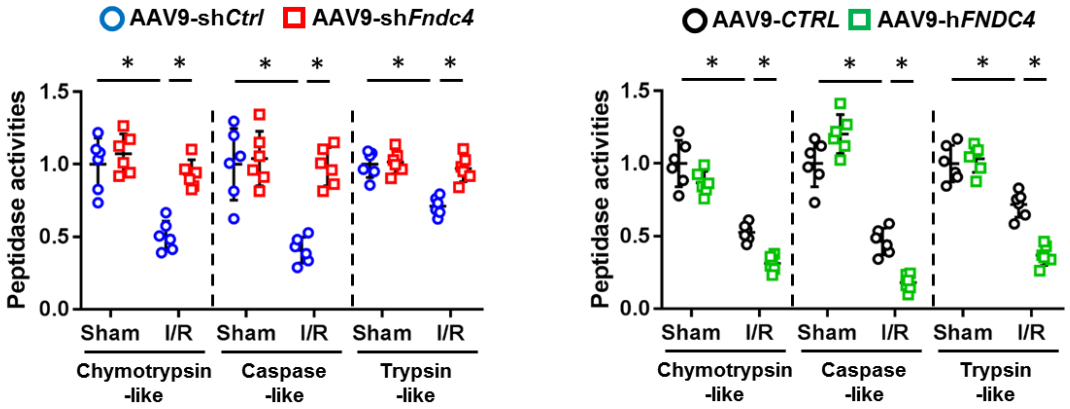
10) If FNDC4 indeed blocks proteasomal degradation, the level of hydroxylated and ubitinated HIF should increase in the cell. Please demonstrate this.

Response: Thanks for your comment. As suggested, we measured the levels of hydroxylated and ubiquinated HIF1α, and found that the hydroxylated and ubiquinated HIF1α levels were dramatically increased in FNDC4-overexpressed NRCMs under sI/R stimulation (Supplementary Figure 13B).



11) What is the effect of FNDC4 on proteasome activity? Please show.

Response: Thanks for your comment. As suggested, we measured the proteasomal activity, and found that FNDC4 overexpression dramatically suppressed, while FNDC4 knockdown restored the proteasomal activity in I/R-stressed hearts (Supplementary Figure 13C).



12) What happens to other endogenous targets of the proteasome? Are they also increased?

Response: Thanks for your comment. The ubiquitin proteasome system is the major protein quality control system in eukaryotic cells, and 80-90% of intracellular proteins were degraded by the proteasome. The endogenous targets of the proteasome are numerous for detection. In our study, we identified that FNDC4 activated HIF1α to prevent cardiac I/R injury, and further studies showed that FNDC4 activated HIF1α through inhibiting its proteasomal degradation. As shown in the revised manuscript, the mRNA levels of genes encoding the components of proteasome, as well as the activities of proteasome, were dramatically inhibited by FNDC4 overexpression. Given these in mind, we did not measure other endogenous targets of the proteasome. We felt really sorry for lacking the data.

13) How does FNDC4 inhibit the proteasome? Please identify the mechanism.

Response: Thanks for your comment. As shown in Figure 7E, the mRNA levels of genes encoding the components of proteasome were dramatically inhibited by FNDC4 overexpression. We indicated the findings in our revised manuscript.

14) VEGF is an important HIF1-dependent gene. Why did VEGF level not increase in this study?

Response: Thanks for your comment. Our findings, including the unbiased transcriptome data, showed that FNDC4 overexpression indeed did not affect VEGF expression despite the activation of HIF1α. We had no data to elucidate this comment currently. We speculated that VEGF expression was also regulated by other transcription factor, and that HIF1α-dependent regulation of VEGF by FNDC4 was offset by other upstream regulators of VEGF.

Reviewer #1 (Remarks to the Author):

This manuscript by Zhang et al investigates roles for FNDC4 in the hearts of mice subjected to cardiac ischemia/reperfusion. This is a very thorough and interesting study where the authors conclude that "...FNDC4 alleviates cardiac I/R injury through facilitating HIF1α-dependent cardiomyocyte survival and angiogenesis. "

For the most part the experiments are well conceived, described and performed, and generally interpreted well. I did not see any flaws in the data or their analyses. Moreover, the authors present sufficient data to demonstrate the viability of the mechanism that they conclude.

Response: Thanks for your comment, and we are grateful for your consideration on our revised manuscript.

Reviewer #3 (Remarks to the Author):

With the revision, the manuscript has partially improved, but concerns remain. Numbers before the paragraphs refer to my previous points.

1) Fig 1N: Even if the authors do not have Kaplan Meyer-Curves, statistics can be applied to this figure and has to be performed.

Response: Thanks for your comment. We felt really sorry that we did not address this comment correctly. **As shown in the figure legends of Fig 1N, $\chi 2$ test was performed for statistical analysis**, and we found that higher plasma FNDC4 level predicted a trend of lower mortality rate, with comparable ratio of loss to follow-up. The description was provided in the manuscript and labelled with YELLOW.

2) Fig 5D: The p-value for VEGFa is 9.48^10-8. Why is that not significant?

Response: Thanks for your comment. As indicated in the methods “Transcriptome analysis”, the differentially expressed genes (DEGs) were defined as those with |Fold Change| ≥ 2 and an adjusted P value < 0.05. Despite the significant Q value, the fold change of Vegfa is 1.82, which less than 2. So, this gene was not selected as the DEGs.

3) scRNAseq after MI: This response is not acceptable. The information is important for the global relevance of the manuscript and has to be included.

Response: Thanks for your comment. As suggested, we re-analyzed our previously published scRNA-seq data in the journal Cell Rep Med (GSE247061)[1], and found that eight FNDCs (including FNDC4) were abundantly expressed in MI-operated murine hearts (Il20rb indicates Fndc6). The role of FNDC1, FNDC3 and FNDC5 in the pathogenesis of cardiovascular diseases has been determined in previous studies (described in the INTRODUCTION). FNDC4, FNDC6 (Il20rb), FNDC7 and FNDC10 were all diffusely expressed in different cardiac cells after MI operation. FNDC5 has been shown to protect the heart against various stresses, including ischemia, sepsis, obesity, diabetes and pressure overload, since its definition as a myokine at 2012 by the journal Nature[2, 3]. In addition, our previous studies also showed that FNDC5 overexpression dramatically prevented doxorubicin- or aging-induced cardiac injury and dysfunction[4, 5]. FNDC4 shows the strongest homology with FNDC5; however, its role in cardiovascular diseases remain elusive. Of note, our unpublished study determined that cardiac-specific overexpression of FNDC4 could prevent cardiac aging in mice (**revised by the journal Cardiovascular Research**). Based on these findings, we thus investigated the role and mechanisms of FNDC4 in cardiac I/R injury. Unlike the increased FNDC4 expression in I/R-stressed hearts, we did not observe any alteration of FNDC4 mRNA in the myocardium after MI. We speculated that the discrepancy might be ascribed to the **different mouse models (ischemia-reperfusion for I/R and permanent ischemia for MI)** and **different detection time (24 h after I/R and 2 weeks after MI)**. Consistent with our hypothesis, previous studies also indicated that the pathophysiologic mechanism might be distinctly different during myocardial ischemia and I/R[6, 7].

[1] Wu Q, Yao Q, Hu T, et al. Dapagliflozin protects against chronic heart failure in mice by inhibiting macrophage-mediated inflammation, independent of SGLT2. Cell Rep Med. 2023;4(12):101334.

[2] Boström P, Wu J, Jedrychowski MP, et al. A PGC1-α-dependent myokine that drives brown-fat-like development of white fat and thermogenesis. Nature. 2012;481(7382):463-8.

[3] Chi C, Fu H, Li YH, et al. Exerkine fibronectin type-III domain-containing protein 5/irisin-enriched extracellular vesicles delay vascular ageing by increasing SIRT6 stability. Eur Heart J. 2022;43(43):4579-4595.

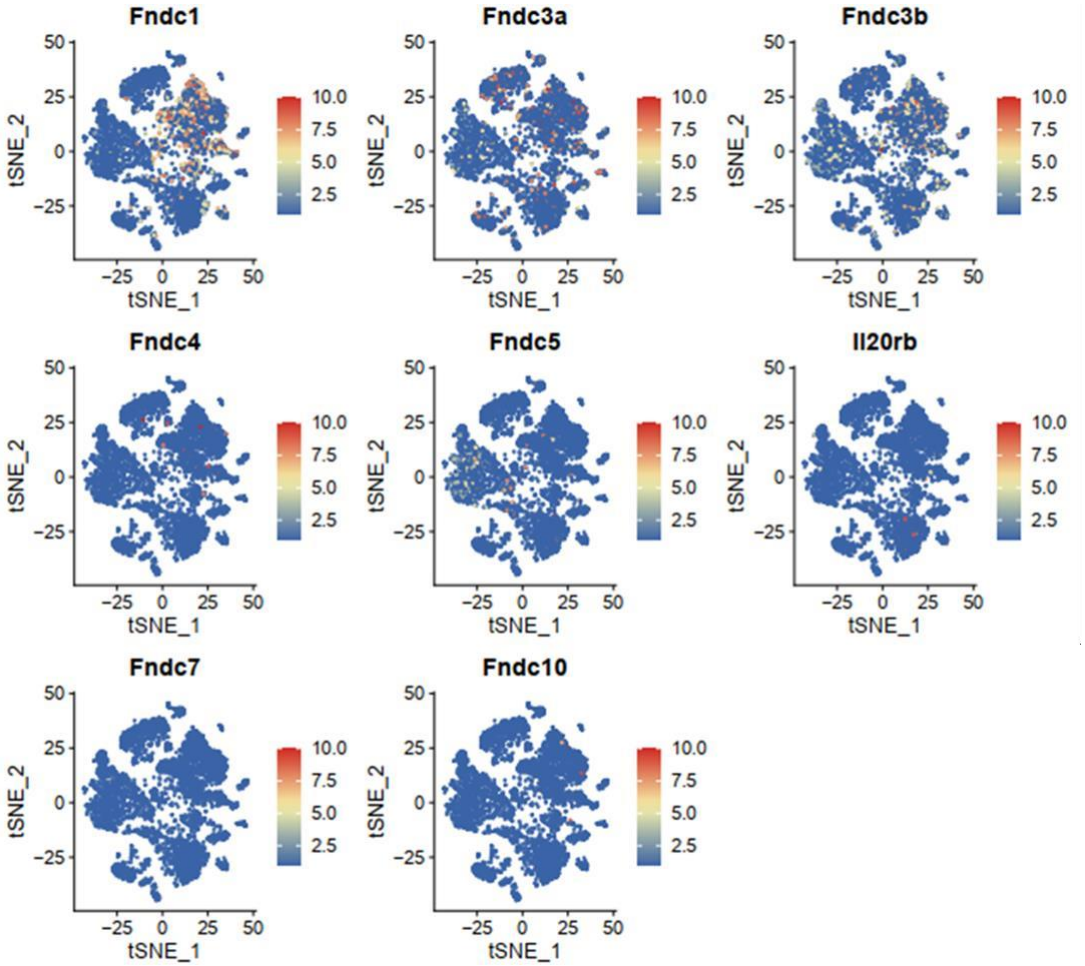
[4] Zhang X, Hu C, Kong CY, et al. FNDC5 alleviates oxidative stress and cardiomyocyte apoptosis in doxorubicin-induced cardiotoxicity via activating AKT. Cell Death Differ. 2020;27(2):540-555.

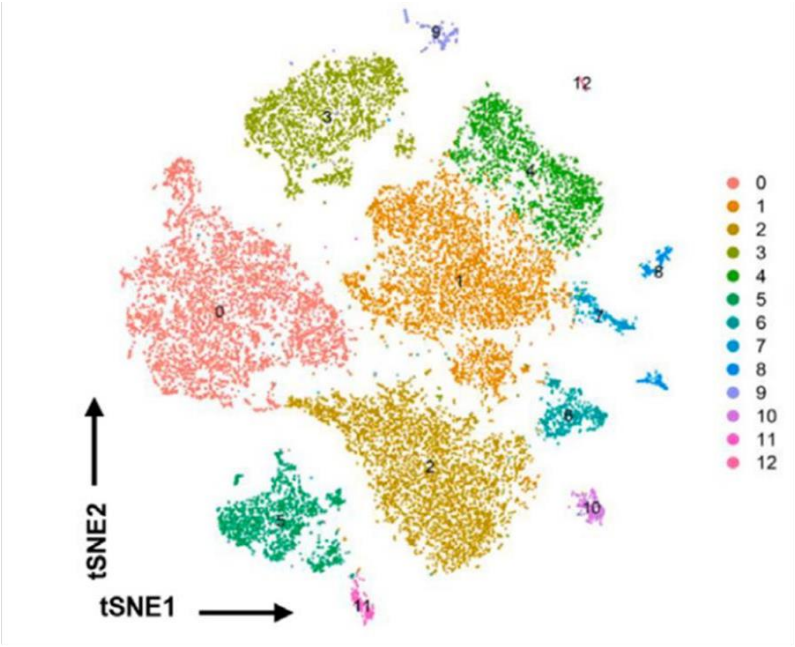
[5] Hu C, Zhang X, Hu M, et al. Fibronectin type III domain-containing 5 improves aging-related cardiac dysfunction in mice. Aging Cell. 2022;21(3):e13556.

[6] Matsui Y, Takagi H, Qu XP, et al. Distinct roles of autophagy in the heart during ischemia and

reperfusion: roles of AMP-activated protein kinase and Beclin 1 in mediating autophagy. Circ Res. 2007;100(6):914-22.

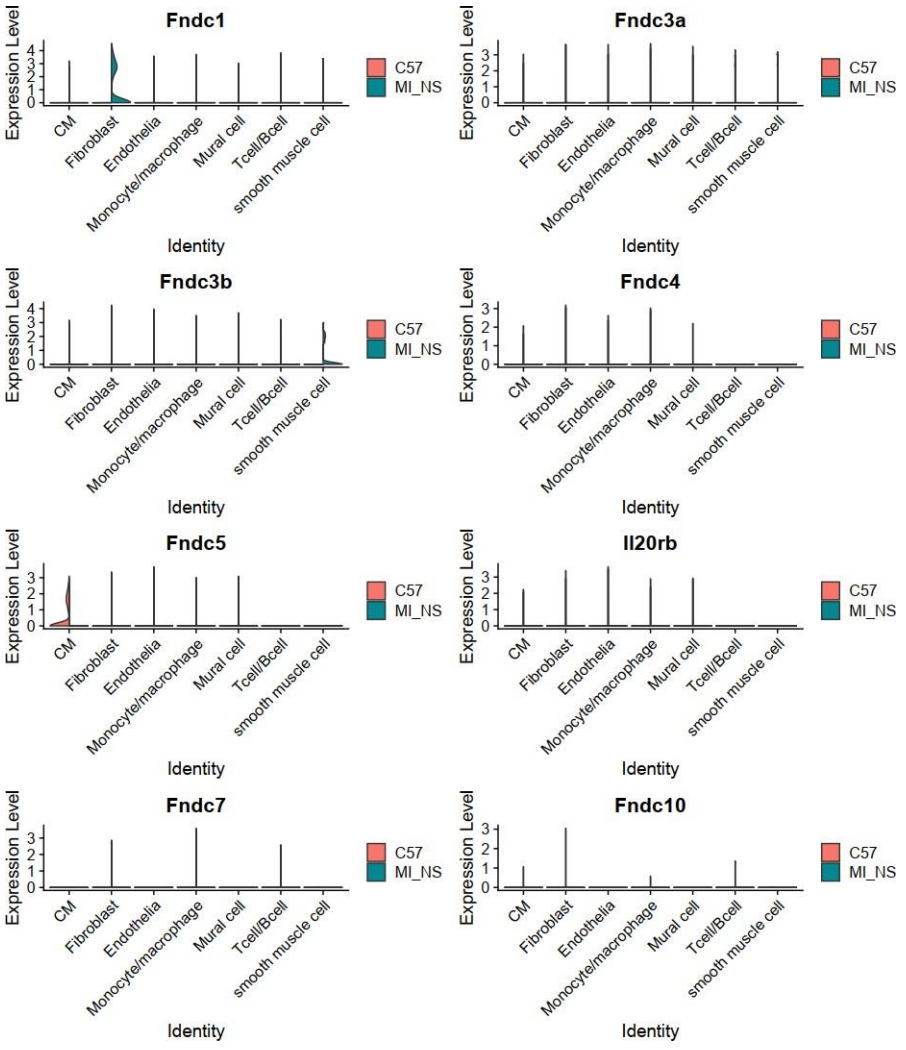
[7] Zhai PY, Sciarretta S, Galeotti J, et al. Differential roles of GSK-3β during myocardial ischemia and ischemia/reperfusion. Circ Res. 2011;109(5):502-11.





Cardiomyocytes: cluster 0
Endothelial cells: clusters 2, 6, 10, 12
B cells: cluster 8

Fibroblasts: clusters 1, 4, 7
Monocytes/macrophages: clusters 3, 9
Smooth muscle cells: cluster 11



4) This point is sufficiently addressed.

Response: Thanks for your comment, and we are grateful for your consideration on our revised manuscript.

5) This point is sufficiently addressed.

Response: Thanks for your comment, and we are grateful for your consideration on our revised manuscript.

6) This point is not sufficiently addressed at all. You are aiming on a journal with a broad audience and therefore you have to expand the focus of the study to increase the appeal. Your statement reads a bit like FNDC4 is like FNDC5. But why should a reader among all these FNDCs be interested specifically in FNDC4, when they are all do the same?

Response: Thanks for your comment. **As indicated in the response to the third comment**, we re-analyzed our previously published scRNA-seq data in the journal Cell Rep Med (GSE247061)[1], and found that eight FNDCs (including FNDC4) were abundantly expressed in MI-operated murine hearts. The role of FNDC1, FNDC3 and FNDC5 in the pathogenesis of cardiovascular diseases has been determined in previous studies (described in the INTRODUCTION). FNDC4, FNDC6 (Il20rb), FNDC7 and FNDC10 were all diffusely expressed in different cardiac cells after MI operation. FNDC5 has been shown to protect the heart against various stresses, including ischemia, sepsis, obesity, diabetes and pressure overload, since its definition as a myokine at 2012 by the journal Nature[2, 3]. In addition, our previous studies also showed that FNDC5 overexpression dramatically prevented doxorubicin- or aging-induced cardiac injury and dysfunction[4, 5]. FNDC4 shows the strongest homology with FNDC5; however, its role in cardiovascular diseases remain elusive. Of note, our unpublished study determined that cardiac-specific overexpression of FNDC4 could prevent cardiac aging in mice (**revised by the journal Cardiovascular Research**). Based on these findings, we thus investigated the role and mechanisms of FNDC4 in cardiac I/R injury.

[1] Wu Q, Yao Q, Hu T, et al. Dapagliflozin protects against chronic heart failure in mice by inhibiting macrophage-mediated inflammation, independent of SGLT2. Cell Rep Med. 2023;4(12):101334.

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[3] Chi C, Fu H, Li YH, et al. Exerkine fibronectin type-III domain-containing protein 5/irisin-enriched extracellular vesicles delay vascular ageing by increasing SIRT6 stability. Eur Heart J. 2022;43(43):4579-4595.

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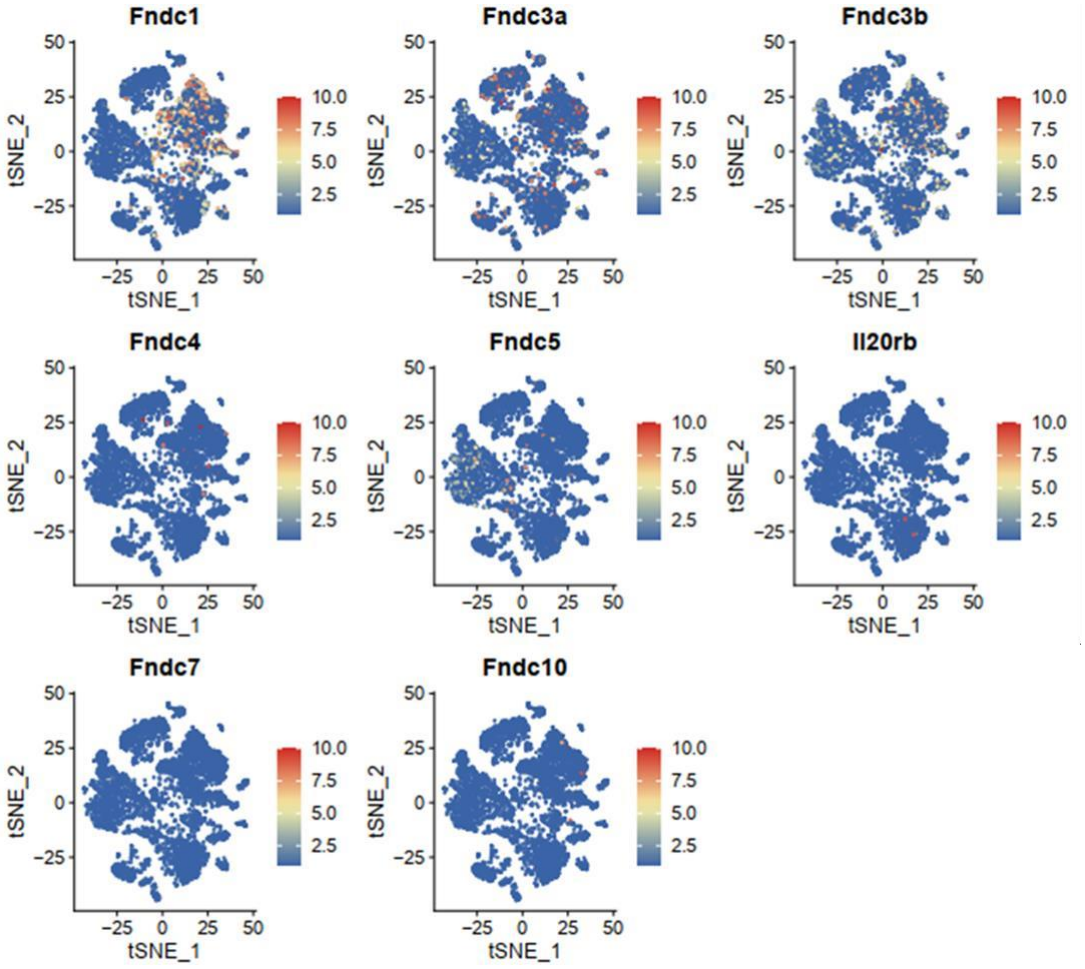
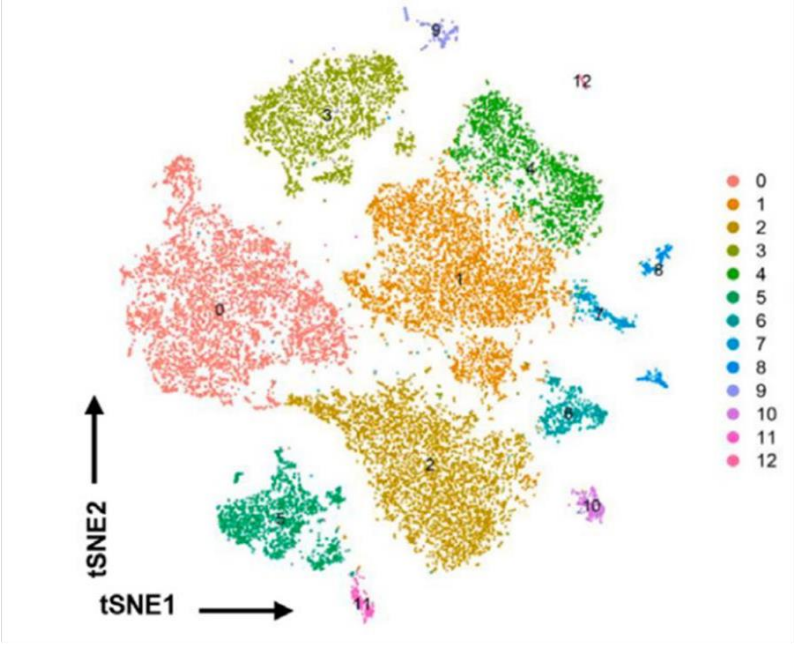




Cardiomyocytes: cluster 0
Endothelial cells: clusters 2, 6, 10, 12
B cells: cluster 8
Fibroblasts: clusters 1, 4, 7
Monocytes/macrophages: clusters 3, 9
Smooth muscle cells: cluster 11

7) This point is sufficiently addressed.

Response: Thanks for your comment, and we are grateful for your consideration on our revised manuscript.

8) Obviously, this response as expected, but it also imposes a big problem to the manuscript. Given that HIF is equally important under both conditions, the proposed effect is unlikely to be specific. In fact, it might not even exists, when the inhibitory effect of PX-478 in the control is identical to the AAV-group. This reviewer does not understand how the authors can exclude this problem? Or put it like this: What is the real evidence that the effect is mediated by HIF?

Response: Thanks for your comment, and we really agree with your opinion. In fact, additional groups (I/R+AAV9-CTRL+PX-478) were included in the original experiments, and we also confirmed the deleterious role of PX-478 in the control-AAV group during analyzing these data. Due to the acknowledged role of PX-478 during cardiac I/R injury, we did not provide the findings in the manuscript last time. As suggested by the reviewer, to address the present comment, we included these data in revised Figure 6D-F, and the data showed that PX-478 dramatically blocked the cardioprotective effects of FNDC4.

9) These authors only provide speculation but not a single experiment to address this crucial point. This is not acceptable.

Response: Thanks for your comment, and we felt really sorry for lacking the experimental data. As we know, protein homeostasis is modulated by various factors, including transcription, post-transcriptional modification, translation and post-translational modification (ubiquitination, acetylation, Neddylation, SUMOylation, etc), and these regulatory mechanisms on HIF1α expression were complete under normoxia, which can compensate for FNDC4-mediated regulation on HIF1α expression[1, 2]. Under I/R stimulation, these regulatory mechanisms are directly modulated by ischemic stimuli, and subsequently cannot compensate for FNDC4-mediated regulation on HIF1α expression[3]. Consistently, various studies in this journal (Nature Communications) or other journals have shown that the expressions of some proteins (including HIF1α) were unaffected under non-stressed conditions, but upregulated or downregulated under different stresses[4-6]. Due to the complex regulation on protein expression, this comment cannot be addressed using a single experiment, but requires subtle projects. This is beyond the scope of the present study. We felt really sorry for lacking the experimental data.

[1] Meijering RA, Henning RH, Brundel BJ. Reviving the protein quality control system: therapeutic target for cardiac disease in the elderly. Trends Cardiovasc Med. 2015; 25(3): 243-7.

[2] Semenza GL. Hypoxia-inducible factor 1: oxygen homeostasis and disease pathophysiology. Trends Mol Med. 2001; 7(8): 345-50.

[3] Calise J, Powell SR. The ubiquitin proteasome system and myocardial ischemia. Am J Physiol Heart Circ Physiol. 2013; 304(3): H337-49.

[4] Wang JY, Lu WT, Zhang J, et al. Loss of TRIM29 mitigates viral myocarditis by attenuating PERK-driven ER stress response in male mice. Nat Commun. 2024; 15(1): 3481.

[5] Chen HM, Chew G, Devapragash N, et al. The E3 ubiquitin ligase WWP2 regulates pro-fibrogenic monocyte infiltration and activity in heart fibrosis. Nat Commun. 2022; 13(1): 7375.

[6] He X, Cantrell AC, Williams QA, et al. p53 Acetylation Exerts Critical Roles in Pressure Overload-Induced Coronary Microvascular Dysfunction and Heart Failure in Mice. Arterioscler Thromb Vasc Biol. 2024; 44(4): 826-42.

10) This point is sufficiently addressed.

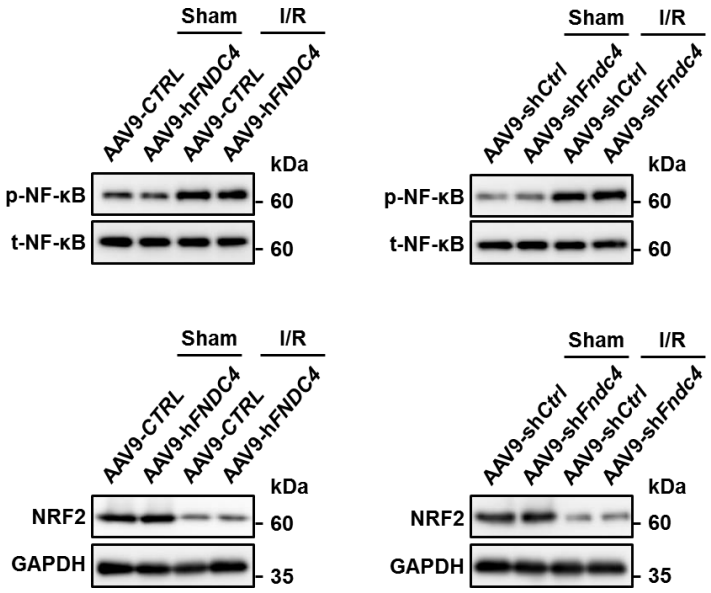
Response: Thanks for your comment, and we are grateful for your consideration on our revised manuscript.

11) This point is sufficiently addressed.

Response: Thanks for your comment, and we are grateful for your consideration on our revised manuscript.

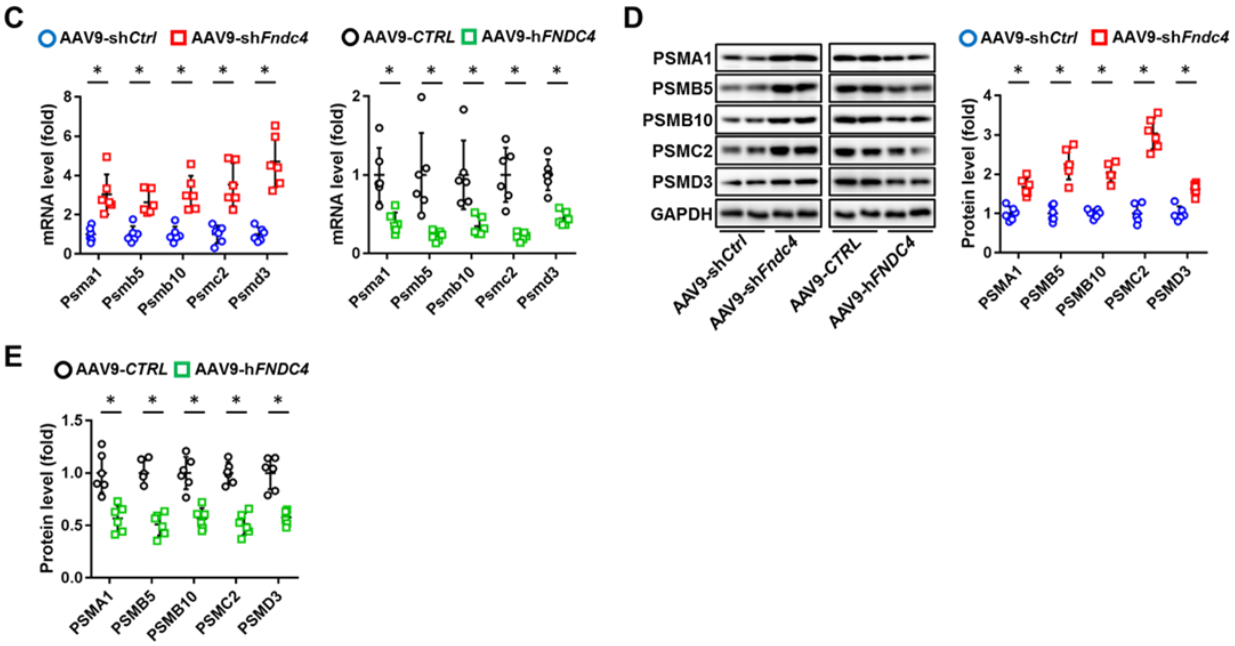
12) With all respect, but have the authors considered that for example NFkappaB and NRF2 are also controlled by the proteasome? Such important transcription factors could also easily contribute to the mechanism suggested here. Such data are required.

Response: Thanks for your comment. As suggested, we detected the expression of NFkappaB and NRF2, and found that their expressions were unaffected by FNDC4 overexpression or knockdown under I/R stress. The data were provided as following. We do not deny that the expressions of these two proteins (NFkappaB and NRF2) are post-transcriptionally regulated by the proteasome. Yet, the protein levels of NFkappaB and NRF2 were indeed unaffected by FNDC4 despite its inhibition on proteasomal degradation. As elucidated in the response to 9th comment, protein homeostasis is modulated at different levels, including transcription, post-transcriptional modification, translation and post-translational modification (ubiquitination, acetylation, Neddylation, SUMOylation, etc), this may partially explain the findings.



13) This is an interesting observation, but the data are incomplete in that respect that they exclusively focus on the overexpression scenario. What happens to the components during I/R with and without overexpression and knockdown respectively? Is the downregulation also apparent on the protein level?

Response: Thanks for your comment. As suggested, we also determined the expressions of proteasomal components in the myocardium under FNDC4 knockdown. Meanwhile, the protein levels of these proteasomal components were also measured. The data were provided in Supplementary Figure 13C-E.



14) Okay, I can understand that this is not a central aspect of the manuscript.

Response: Thanks for your comment, and we are grateful for your consideration on our revised manuscript.

Reviewer #4 (Remarks to the Author):

Thank you for your invitation.

The manuscript by Zhang et al. investigated the protective role of FNDC4 in heart under ischemia/reperfusion (I/R) in mice. And mechanically, FNDC4 promotes cardiomyocyte survival and angiogenesis through HIF1α dependent pathway.

The author's responses to most of the questions raised by Reviewer 2 were sufficient. But in the answer to to Question 3, the author should show the body weight data of mice receiving AAV9-hFNDC4, AAV9-shFndc4 or rFNDC4 treatment, which would answer the reviewer's question perfectly.

Response: Thanks for your comment. As suggested, the body weight was described in the revised manuscript, and the information was labelled with RED in the RESULTS sections "Cardiac-specific FNDC4 overexpression facilitates cardiomyocyte survival and angiogenesis during cardiac I/R injury", "Cardiac-specific FNDC4 knockdown inhibits cardiomyocyte survival and angiogenesis during cardiac I/R injury" and "Therapeutic administration of rFNDC4 protein is sufficient to attenuate cardiac I/R injury".

I believe that investigating the universal or specific receptors of FNDC4 in heart and how FNDC4 is secreted to extracellular space would help us better understand the role of FNDC4 in cardiac pathophysiology.

Response: Thanks for your insightful comment, and we really agree with your opinion that investigating the receptors and cleavage mechanisms of FNDC4 would help us better understand the role of FNDC4 in cardiac pathophysiology. Among all FNDCs proteins, FNDC4 displays a high homology with FNDC5. Bosma et al. previously determined that FNDC4 was cleaved at the C-terminus as FNDC5, and that the N-terminal fragment was released an extracellular portion of the protein. In addition, previous studies have determined the receptor-dependent and receptor-independent role of FNDC5, so whether and which specific receptors were involved in the cardioprotective effects of FNDC4 deserve meticulous investigation. Georgiadi et al. previously identified GPR116 as a receptor of FNDC4 in the white adipose tissue[1]. However, our study revealed that endothelial cells were neither the origin nor the direct target of FNDC4. Moreover, Niaudet et al. found that GPR116 knockout dramatically increased vascular density, which was contrary to our findings showing that FNDC4 facilitated angiogenesis in I/R-stressed hearts[2]. Based on these studies, we did not explore the involvement of GPR116 in FNDC4-mediated cardioprotection against I/R injury. We have added these in our revised DISCUSSION. As suggested, we discussed this in our manuscript, and the information was labelled with YELLOW.

[1] Georgiadi A, Lopez-Salazar V, Merahbi RE, et al. Orphan GPR116 mediates the insulin sensitizing effects of the hepatokine FNDC4 in adipose tissue. Nat Commun. 2021;12(1):2999.

[2] Niaudet C, Petkova M, Jung B, et al. Adgrf5 contributes to patterning of the endothelial deep layer in retina. Angiogenesis. 2019;22(4):491-505.