Peer Review File

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Reviewer A

How did the authors decide upon 5 days after RIR injury as their study endpoint? Did they consider investigating at longer durations after RIR injury?

Reply 1: Thank you for your helpful comments.

In our previous study, compared with control, we performed retinal flat mounts and detected that the number of RGC remarkably reduced in the RIR treated retinas on the fifth day(1). In this study, the number of RGC significantly decreased in LCN2-TG mice compared with wild-type (WT) mice on the fifth day after RIR injury. We decided upon 5 days after RIR injury as our study endpoint.

As you suggested, we performed retinal flat mounts on the seventh day after RIR injury to assess the extent of RGC injury. Our data showed that the number of RGC significantly reduced and almost all of them died in LCN2-TG mice compared with WT mice (see below figure A, B).

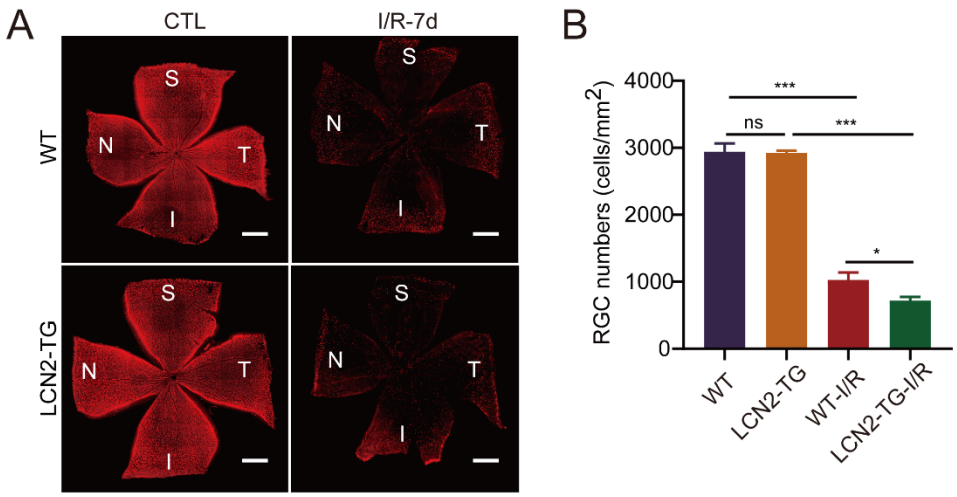


Figure A, B. TissueFaxs images of retinal flat mounts showed that the number of RGC in whole retina.

(A) TissueFaxs images of retinal flat mounts showed that the number of RGC in retina on the seventh day after RIR injury. RGCs were immunostained with an anti-RBPMS antibody. Scale bar: 500 μm. CTL: control. S, superior; I, inferior; N, nasal; and T, temporal.

(B) Quantification of RGC performed for 200 × 200 μm area in 4 quadrants from the peripheral, middle and central retina and averaged for retinas per each control and experimental condition. CTL group, n = 3 mice per group. I/R group, n=6 mice per group. All data represent mean ± SEM. *P < 0.05, ***P < 0.001, ns: no significance.

Reviewer B

The manuscript by Mei et al. entitled “Lipocalin 2 induced visual impairment by promoting ferroptosis in retinal ischemia/reperfusion injury” demonstrates a link between the elevated expression level of LCN2 protein and retinal ganglion cell (RGC) death in elevated intraocular pressure (IOP)-induced retinal ischemia/ reperfusion (RIR) injury model. Furthermore, the authors suggest LCN2 promotion of ferroptosis as a possible mechanism in the pathogenesis of ischemic retinopathy.

The relationship between LCN2 and ferroptosis has been extensively reported in humans and mice in the contexts of cancer and inflammatory diseases (including diseases of the eyes). LCN2 has also been shown to increase RGC cell death. This paper connects these bodies of work, showing a relationship between LCN2 to ferroptosis-mediated RGC death. Although there are some typos, grammatical errors, and room for clarity (see the details below), overall the manuscript is concise and readable.

However, I do have several major and minor comments (see below) that should be addressed to enhance the quality of the manuscript.

Major comments:

1. The authors claim, at the beginning of the conclusion section (line 358), that “LCN2 is a critical regulator in promoting RGC death ….”. This claim is not substantiated by the data and showed be toned down here and throughout the manuscript. The study shows correlations of elevated LCN2 level, ferroptosis, and RGC death in the IOP-induced RIR injury model. During the non-injury condition (control), there is no difference between WT and the overexpression model (LCN2-TG-expressing human as well as mice LCN2). The elevated LCN2 level alone does induced RGC death, even with elevated levels of inflammatory cytokines and chemokines (Fig. 4) in absence of injury. During the IOP-induced RIR, there are elevated levels of cell death and inflammatory response in LCN2-TG compared to WT. To claim LCN2 as a critical regulator of the process, the authors should include data showing the absence or substantially decreased RGC cell death in LCN2 knock-out mice also. The LCN2 knock-out line has already been reported in many previous studies, so it is unclear why the authors do not show results using the knock-out line. Comparison of WT with knock-out and over expression is vital to claim that LCN2 is a “critical regulator”.

Reply 1: Thank you for your thorough review and positive evaluation.

As we haven’t enough LCN2 knockout mice, we performed experiments with LCN2 heterozygous ($LCN2^{+/-}$) mice to investigate the effect of LCN2 deletion on RGC death in the IOP-induced RIR injury model. Compared with control, RGC death was alleviated and visual function was restored in $LCN2^{+/-}$ mice after RIR injury (see below for details).

Since overexpressed LCN2 resulted in retinal ferroptosis, and aggravated RGC death and visual impairment after RIR injury, we performed LCN2 heterozygous mouse to investigate whether downregulation of LCN2 could alleviate RGC loss. Targeted allele was knocked out in exons 1~5 of *Lcn2* on mouse chromosome 2 via CRISPR/Cas9 editing system (Figure 6A). Compared with WT mice, protein level of LCN2 decreased by 50% in the retinas of $LCN2^{+/-}$ mice (Figure 6B). The number of RGC in retina were significantly increased in $LCN2^{+/-}$ mice after RIR injury (Figure 6C, 6D). Moreover, the impairment of visual function could be remarkably ameliorated in $LCN2^{+/-}$ mice treated with RIR injury (Figure 6E, 6F). Taken together, these results showed that RGC death was alleviated and visual function was restored in $LCN2^{+/-}$ mice.

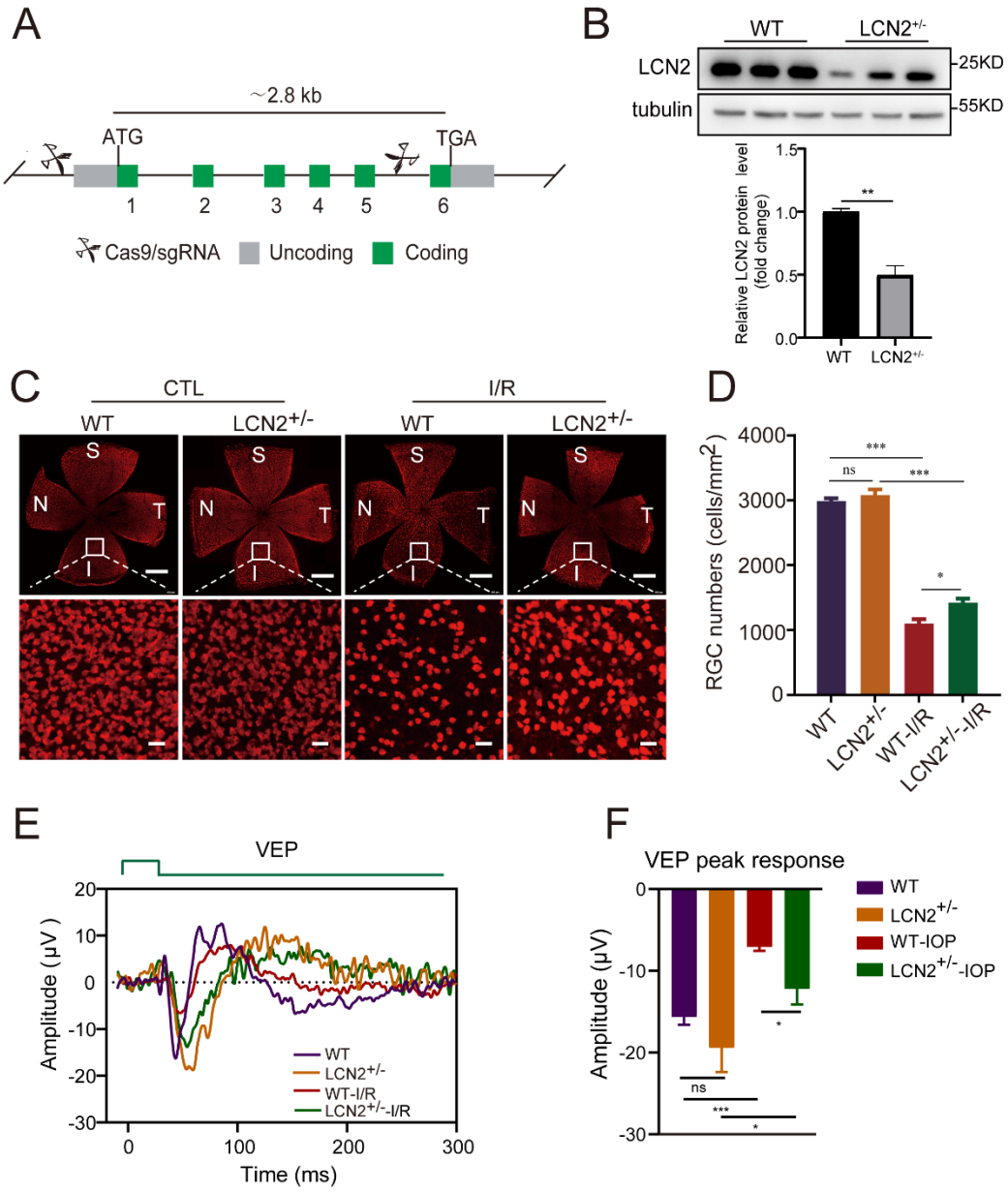


Figure 6. Downregulated LCN2 can alleviate RGC death and retinal impairment in RIR injury

(A) Schematic diagram of LCN2 knockout mice generated at exons 1~5 of *Lcn2* on mouse chromosome 2 via CRISPR/Cas9 system.

(B) The protein level of LCN2 in retinas of LCN2$^{+/-}$ mice were detected by western blotting. n = 3 mice per group. All data represent mean ± SEM. **P < 0.01.

(C) TissueFaxs images of retinal flat mounts showed that the number of RGC in retina. RGCs were immunostained with an anti-RBPMS antibody. Scale bar: 500 μm (top). Scale bar: 20 μm (bottom). CTL: control. S, superior; I, inferior; N, nasal; and T, temporal.

(D) Quantification of RGC performed for 200 × 200 μm area in 4 quadrants from the peripheral, middle and central retina and averaged for retinas per each control and experimental condition. n = 4 mice per group. All data represent mean ± SEM. *P < 0.05, ***P < 0.001, ns: no significance.

(E, F) Visual evoked responses (VEPs) of WT and LCN2$^{+/-}$ mice in left eyes (without RIR injury) and in right eyes (with RIR injury). Shown in (E) are response and (F) are amplitudes

of the VEPs response peaks. n = 5 mice per group. All data represent mean ± SEM. *P < 0.05, ***P < 0.001, ns: no significance. Statistical analysis was performed with unpaired two-tailed student's t-test.

Changes in the text: we added some data in our manuscript (see results section, page 18, line 351 to 364, see figure legends, page 30 to 31, line 651 to 669, and see figure 6).

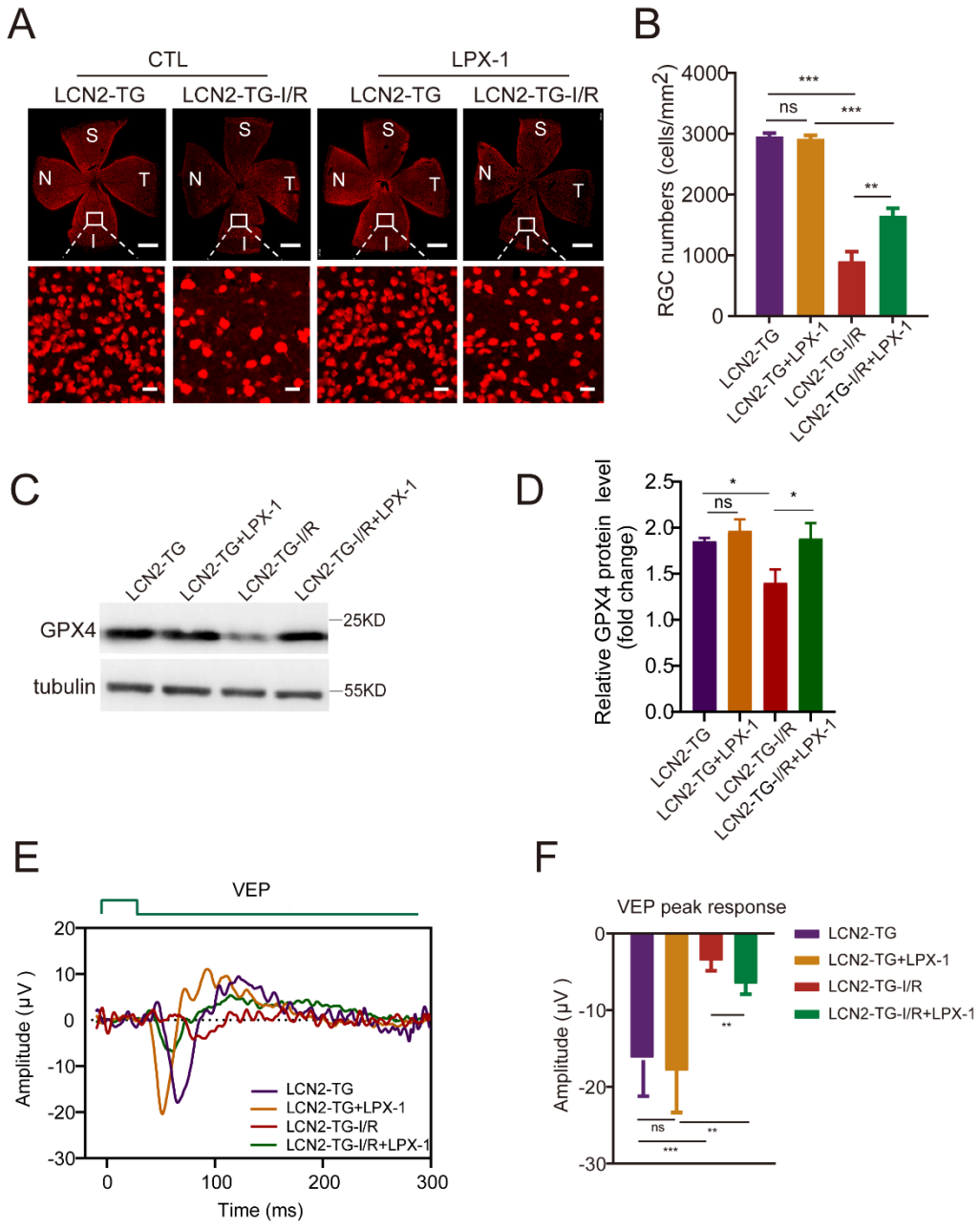
2. The data from Figure 5 is used to claim that LCN2 induced ferroptosis can be inhibited by liproxystatin-1 (Line 311). However, the overexpression line (LCN2-TG) is not included in these experiments. If the authors statement is true, I would expect that treatment of liproxstatin-1 should be able to rescue RGCs death in LCN2-TG-I/R also (not only in WT I/R). This would support that hypothesis that LCN2 promotes ferroptosis induced RGCs death. In addition, the liproxstatin-1 treatment experiments in the LCN2 knock-out background would provide further support for this statement.

Reply 2: Thank you for your helpful comments and suggestions.

We conducted experiments with treatment of liproxstatin-1 in LCN2-TG-I/R mice to rescue RGCs death. Our data showed that the treatment of liproxstatin-1 could rescue RGC death and vision function impairment in LCN2-TG-I/R mice (see below for details).

To investigate the treatment effect of liproxstatin-1 in LCN2-TG-I/R, we used inhibitors of ferroptosis (liproxstatin-1, LPX-1) to rescue RGC death and visual impairment. Compared with LCN2-TG mice, intraperitoneal treatment with LPX-1 (10 mg/kg body weight) of LCN2-TG mice could effectively ameliorate RGC death after RIR injury (Supplementary Figure 6A, 6B). Also, in RIR injured LCN2-TG mouse retinas, intraperitoneal LPX-1 treatment led to significantly increased protein levels of GPX4, which indicated that retinal ferroptosis was inhibited by LPX-1-treatment (Supplementary Figure 6C, 6D). Moreover, compared with control mice, apparently higher VEPs response could be triggered in LPX-1-treated mice (Supplementary Figure 6E, 6F). These findings demonstrated that LCN2 induced ferroptosis could be inhibited by liproxstatin-1 in LCN2-TG mice, and inhibiting retinal ferroptosis could efficiently restore RGC death and vision function impairment in RIR injury model.

Sufficient LCN2 knockout mice were obtained in the future, we will perform experiments with treatment of liproxstatin-1 in LCN2 knockout mice to further confirm.



Supplementary Figure 6. Overexpressed LCN2 induced RGC damage can be ameliorated by ferroptosis inhibitor

(A) TissueFaxs images of retinal flat mounts showed that the number of RGC in retina. RGCs were immunostained with an anti-RBPMS antibody. Scale bar: 500 μm (top). Scale bar: 20 μm (bottom). CTL: control. S, superior; I, inferior; N, nasal; and T, temporal.

(B) Quantification of RGC performed for 200 × 200 μm area in 4 quadrants from the peripheral, middle and central retina and averaged for retinas per each control and experimental condition. n = 6 mice per group. All data represent mean ± SEM. **P < 0.01, ***P < 0.001, ns: no significance.

(C, D) The protein level of GPX4 in retinas were detected by western blotting. n = 3 mice per group. All data represent mean ± SEM. *P < 0.05, ns: no significance.

(E, F) Visual evoked responses (VEPs) of LCN2-TG and LCN2-TG+LPX-1 mice in left eyes

(without RIR injury) and in right eyes (with RIR injury). Shown in (E) are representative individual response and (F) are amplitudes of the VEPs response peaks. n = 5 mice per group. All data represent mean ± SEM. **P < 0.01, ***P < 0.001, ns: no significance. Statistical analysis was performed with unpaired two-tailed student's t-test.
Changes in the text: we added some data in our manuscript (see results section, page 17 to 18, line 338 to 347, and see supplementary figure 6).

Minor comments:
1. In the abstract, it says that "A mouse model of elevated intraocular pressure (IOP)-induced retinal ischemia/reperfusion injury was established." But in the methods, there is a citation, indicating that this is not a new model. The word "established" is mis-leading, unless this is a new model. If this is a new model system, then the generation of this model should be described in the main text.
Reply 1: Thank you for your helpful suggestions.
Changes in the text: We have replaced "A mouse model of elevated intraocular pressure (IOP)-induced retinal ischemia/reperfusion injury was established" with "A mouse model of elevated intraocular pressure (IOP)-induced retinal ischemia/reperfusion injury was performed" in our manuscript (see abstract section, page 3, line 35 to 36).

2. Overall, there are inconsistencies in the naming of genes and proteins. Gene symbols are italicized, with only the first letter in upper-case (e.g., Lcn2). Protein symbols are not italicized, and all letters are in upper-case (e.g., LCN2). This should be corrected throughout the manuscript and supplementary data (primers for the genes), figure legends, etc.
Reply 2: Thank you for your helpful suggestions.
Changes in the text: We have corrected the naming of genes and proteins in our manuscript as advised.

3. Overall, the fluorescent images appear dark and are difficult to interpret. The figures are also a little bit blurry.
Reply 3: Thank you for your helpful suggestions.
Changes in the text: We have adjusted the format of fluorescent images from CMYK to RGB in our manuscript (see figure 1E and figure 4A, 4B).

4. Line 80, "that belongs to" instead of "and belongs to"
Reply 4: Thank you for your helpful suggestions.
Changes in the text: We have corrected "and belongs to" to "that belongs to" in our manuscript (see introduction section, page 6, line 83).

5. Line 110, "for" instead of "at"
Reply 5: Thank you for your helpful suggestions.
Changes in the text: We have corrected "at" to "for" in our manuscript (see methods section, page 7, line 113).

6. Line 111, Rephrase as "all animal procedures were conducted according to"

Reply 6: Thank you for your helpful suggestions.
Changes in the text: We have corrected "all animal procedures according to" to "all animal procedures were conducted according to" in our manuscript (see methods section, page 7, line 113 to 114).

7. Line 116, Rephrase the title as "elevated intraocular pressure-induced retinal ischemia/reperfusion injury model"
Reply 7: Thank you for your helpful suggestions.
Changes in the text: We have corrected "retinal ischemia/reperfusion injury model" to "elevated intraocular pressure-induced retinal ischemia/reperfusion injury model" in our manuscript (see methods section, page 7, line 118 to 119).

8. Line 121, "local" instead of "locally"
Reply 8: Thank you for your helpful suggestions.
Changes in the text: We have corrected "locally" to "local" in our manuscript (see methods section, page 7, line 124).

9. Line 154, "Nuclei" instead of "Nucleus"
Reply 9: Thank you for your helpful suggestions.
Changes in the text: We have corrected "Nucleus" to "Nuclei" in our manuscript (see methods section, page 9, line 157).

10. Line 199, rephrase as "membranes were incubated with corresponding"
Reply 10: Thank you for your helpful suggestions.
Changes in the text: We have corrected "membranes were incubated corresponding" to "membranes were incubated with corresponding" in our manuscript (see methods section, page 11, line 213 to 214).

11. Line 199-202, rephrase the following: "and the blots were showed with "
Reply 11: Thank you for your helpful suggestions.
Changes in the text: We have corrected "and the blots were showed" to "and the blots were showed with" in our manuscript (see methods section, page 12, line 215).

12. Line 214, rephrase the following: "Data were showed"
Reply 12: Thank you for your helpful suggestions.
Changes in the text: We have corrected "Data were showed" to "Data were showed" in our manuscript (see methods section, page 12, line 229).

13. Section starting with line 220, "expression" is a term used for genes. Western blots show protein levels. So "expression" should not be used in this paragraph.
Reply 13: Thank you for your helpful suggestions.
Changes in the text: We have corrected "Western blot analysis showed that the expression of Lcn2" to "Western blot analysis showed that protein level of LCN2" in our manuscript (see results section, page 13, line 240).

14. Line 230, “inserted” instead of “insered”
Reply 14: Thank you for your helpful suggestions.
Changes in the text: We have corrected “insered” to “inserted” in our manuscript (see results section, page 13, line 245).

15. Line 258, delete or further explain the term “newly”
Reply 15: Thank you for your helpful suggestions.
Changes in the text: We have corrected “Ferroptosis is a newly form of” to “Ferroptosis is a form of” in our manuscript (see results section, page 15, line 285).

16. Line 268. rephrase “level were showed”
Reply 16: Thank you for your helpful suggestions.
Changes in the text: We have corrected “levels were showed” to “level were showed” in our manuscript (see results section, page 15, line 297).

17. Line 277, rephrase for clarity (“and exacerbates?”)
Reply 17: Thank you for your helpful suggestions.
Changes in the text: We have corrected “exacerbates” to “and exacerbates” in our manuscript (see results section, page 16, line 310).

18. Line 297, “iron-dependency” instead of “iron-dependent”
Reply 18: Thank you for your helpful suggestions.
Changes in the text: We have corrected “iron-dependent” to “iron-dependency” in our manuscript (see results section, page 17, line 333).

19. Line 303- 305, define “RBPMS” and explain how the staining distinguishes between dead and alive cells.
Reply 19: Thank you for your helpful suggestions. RBPMS (RNA binding protein with multiple splicing) contains one RRM (RNA recognition motif) domain and belongs to the RRM family of RNA-binding proteins. RBPMS has been previously identified as a specific marker for RGC(2). In our experiment, all eyeballs were obtained and fixed with 4% paraformaldehyde for 40 minutes, and then stained with RBPMS. we could not distinguish between dead and alive cells.
Changes in the text: We have modified our manuscript as advised (see results section, page 14, line 267 to 270).

20. Line 308, compared to “control” instead of “WT” because all experiments have been done in WT with LPX-1 treatment or without LPX-1 treatment (control).
Reply 20: Thank you for your helpful suggestions.
Changes in the text: We have corrected “WT” to “control” in our manuscript (see results section, page 17, line 345).

21. Line 318, “leads” instead of “lead”

Reply 21: Thank you for your helpful suggestions.
Changes in the text: We have corrected "lead" to "leads" in our manuscript (see discussion section, page 19, line 369).

22. Line 322 and 324, there are extra spaces before the citations.
Reply 22: Thank you for your helpful suggestions.
Changes in the text: We have removed extra spaces before the citations in our manuscript (see discussion section, page 19, line 368 to 369).

23. Line 329, "damaging" instead of "damaged"
Reply 23: Thank you for your helpful suggestions.
Changes in the text: We have corrected "damaged" to "damaging" in our manuscript (see discussion section, page 19, line 380).

24. Line 331, should say "A recent study…"
Reply 24: Thank you for your helpful suggestions.
Changes in the text: We have corrected "recent study…" to "A recent study…" in our manuscript (see discussion section, page 20, line 394).

25. In the discussion section, please add some reasons, speculations, or possible hypotheses why elevated LCN2 level alone does not exert any effect, for example, does LCN2 work in conjunction with the injury-derived metabolites/ chemicals/ signaling molecules, etc?
Reply 25: Thank you for your helpful suggestions.
Although the distribution of LCN2 in the retina is obviously different between WT and LCN2-TG mice, there is no difference in retinal morphology between two groups before RIR treatment. However, compared with WT mice, the retinas of LCN2-TG mice exhibit more grievous damage with RIR treatment. In LCN2-TG mice, the number of RGC and total retinal thickness are significantly decreased on the fifth day after RIR treatment. Vision function impairment was much severer in LCN2-TG than controls in the RIR model as well. A study reported that NF-κB and STAT1 might be act together as a key promoter-binding complex for activating the expression of LCN2 in the retina at the onset of the disease state(3). Therefore, LCN2 might be served as a stress-responsive gene and it worked in conjunction with the injury-derived signaling molecules in RIR injury model.
Changes in the text: We have modified our manuscript as advised (see discussion section, page 19 to 20, line 382 to 393).

26. Fig 1E, show the fluorescence quantification measurements with standard deviation in order to claim substantial upregulation of LCN2 protein. A graph can be included in the main figure or as a supplementary figure.
Reply 26: Thank you for your helpful suggestions.
Changes in the text: We added some data "LCN2 fluorescence quantification measurements in the retinal ganglion cell layer are shown in figure 1F, and a graph is included in the main figure" in our manuscript. (see results section, page 13, line 253 to 255, see figure 1F and figure legends, page 27, line 580 to 582).

27. Figure 2A, letters denoting layers of retinal are blurred in white color, use an appropriate color that stands out.
Reply 27: Thank you for your helpful suggestions.
Changes in the text: We have corrected the letters denoting layers of retinal from white color to black color in figure 2A (see figure 2A).

28. Fig 2 legend, detail whether the graph in 2E is averages of n=6 or representative individuals.
Reply 28: Thank you for your helpful suggestions.
Changes in the text: We have corrected "the graph are responses" to "the graph is representative individual response" in figure 2E (see figure legends section, page 28, line 599 to 600).

29. Fig 4. quantify GFAP, IBA1, and CD68 fluorescence levels and include measurement and standard deviations in the main or supplementary figure in order to claim substantial upregulated expression.
Reply 29: Thank you for your helpful suggestions.
Changes in the text: We added some data "CRALBP and GFAP fluorescence quantification measurements are shown in supplementary figure 4A, IBA1 and CD68 fluorescence quantification measurements are shown in supplementary figure 5A, and two graphs are included in the supplementary figures" in our manuscript (see supplementary figure 4A and supplementary figure 5A).

30. Fig 4A, add comments about the substantial increase/spread of GFAP expression spatial domain in I/R LCN2-TG at the proper place in the results section.
Reply 30: Thank you for your helpful suggestions.
Changes in the text: We added some data "Double immunology staining for CRALBP and GFAP was used to detect the activation of Müller cells in retinas. Our data showed that elevation of LCN2 promoted the activation of Müller cells in LCN2-TG mice compared with WT mice after RIR injury" (see results section, page 16, line 314 to 318, and see figure 4A).

31. IBA1 results are not discussed in the Fig. 4 legend.
Reply 31: Thank you for your helpful suggestions.
Changes in the text: We have added the results of IBA1 in figure 4 legend as advised (see figure legends section, page 29, line 625 to 626).

Reviewer C

Mei et al have tried to decipher the role of LCN2 in ferroptosis induction in retinal ischemia/reperfusion injury. There are some concerns as follows:

Comments:
1: In the LCN-2 transgenic mice, the expression of the protein is only seen in RGCs. However, several reports have suggested the expression of LCN-2 is present both in astrocytes and in

RPE cells. The authors should check the expression of the protein in these other cell types by double immunology staining on sections. If they are expressed in the other cell types then the effect of LCN-2 in RGC cells specifically and its subsequent role in Ischemia/repercussion injury should not be true and might be the global effect from this inflammatory protein being expressed by these cell types. The authors can also think of generating RGC cell specific transgenic mice to ascertain the role of the protein in RGC signaling pathways.

Reply 1: Thank you for your constructive suggestions.

We used immunostaining of LCN2 to examine its distribution in mice retinas. In the retina of wild type (WT) mice, LCN2 was mainly expressed in the ganglion cell layer (GCL) (Figure 1E). However, in the retina of LCN2-TG mice, LCN2 was not only expressed in the GCL, but also expressed in the retinal other layers (Figure 1E). Therefore, the effect of LCN2 on RGC damage couldn't be excluded as a global effect from LCN2 was expressed by other cell types. We will generate RGC cell specific transgenic mice to ascertain the role of LCN2 in RGC signaling pathways in the future.

Changes in the text: We have corrected our manuscript as advised (see results section, page 13, line 248 to 255).

2. Much more in-depth studies must be done to ascertain the ferroptosis activation status in the RGC cells. Specific ferroptosis markers like FTH1 as well as iron levels in the RGC cells must be done. Treating with LCN-2 shRNA (can be given intravitreally and should contain a reporter with RGC specific promoter) should be used to prove the LCN-2 dependent ferroptosis signaling pathway in the transgenic mice.

Reply 2: Thanks a lot for your constructive advice.

Both ferritin heavy chains (FTH) and ferritin light chains (FTL) can be regarded as markers of ferroptosis(4, 5). To further ascertain the ferroptosis activation status in the retina, we used ferritin light chains (FTL) staining of retinal sections to assess the level of FTL by immunofluorescence. The level of FTL decreased sharply after RIR injury in LCN2-TG mice compared with WT mice (Figure 3E, 3F).

Retinal iron levels were detected using iron assay kit (abcam, ab83366). We failed to detect iron levels in the retinas of both WT and LCN2-TG mice, presumably because of insufficient sample size.

We will verify the role of LCN2 in ferroptosis signaling pathway according to your valuable advice in the future.

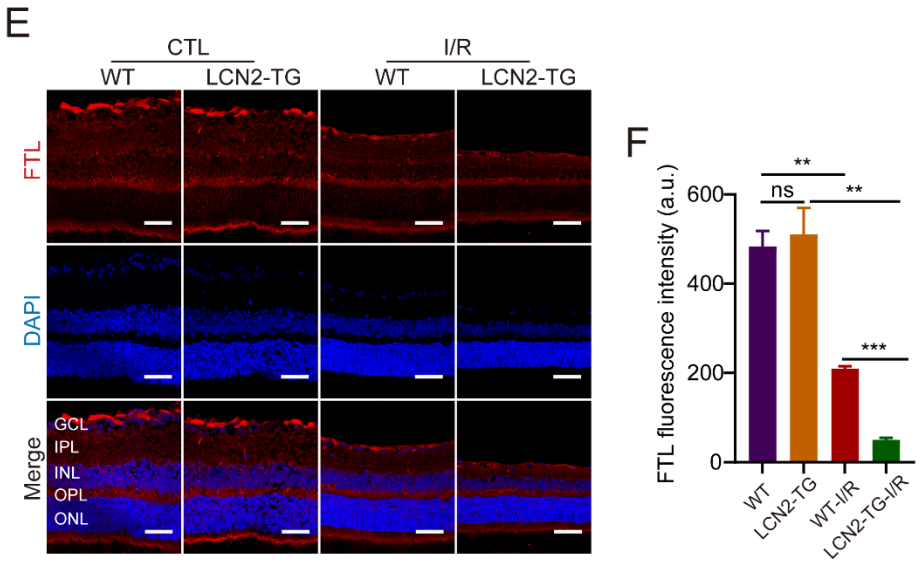


Figure 3. Upregulated LCN2 facilitates retinal ferroptosis in RIR injury model.
(E, F) Representative photomicrographs of immunofluorescence staining for FTL (red) in the retina. n = 3 mice per group. All data represent mean ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001, ns: no significance. Statistical analysis was performed with unpaired two-tailed student's t-test.

Changes in the text: We added some data in our manuscript as advised (see results section, page 15 to 16, line 302 to 306, see figure legends section, page 29, line 615 to 619, and see figure 3E, 3F).

3. Muller cell activation must be confirmed with double staining with CRALBP/GFAP and confirm the role/presence of glial activation in the WT+I/R and TG+I/R mice.

Reply 3: Thanks a lot for your constructive advice.

Double immunology staining for CRALBP and GFAP was used to detect the activation of Müller cells in retinas. Our data showed that elevation of LCN2 promoted the activation of Müller cells in LCN2-TG mice compared with WT mice after RIR injury (Figure 4A).

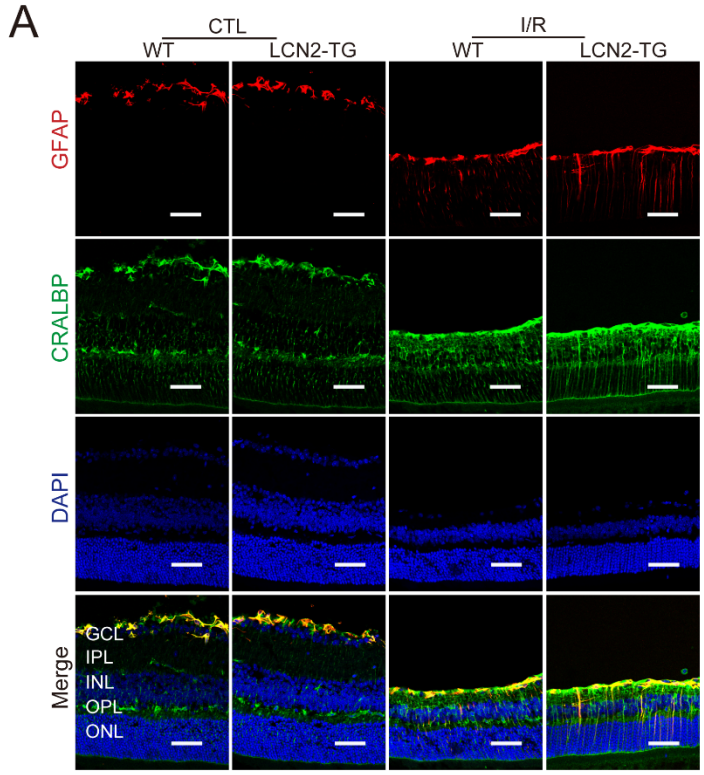


Figure 4. Upregulated LCN2 promotes activated glial cells and aggravates inflammatory responses.

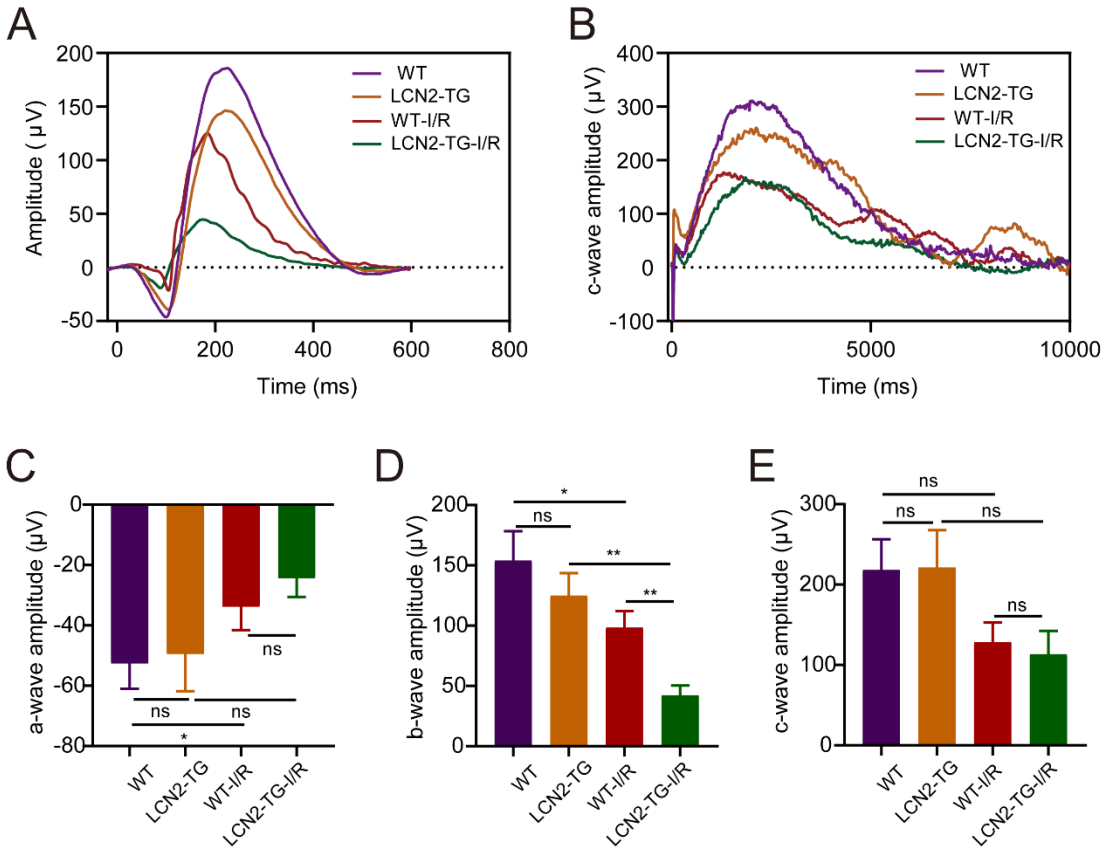
(A) Double immunology staining for CRALBP (green) and GFAP (red) in the retinal sections from CTL or RIR injury group. Scale bar: 50 μm. CTL: control.

Changes in the text: We have corrected figure 4A as advised (see results and figure legends section, page 16, line 314 to 318, page 29, line 622 to 624 and see figure 4A).

4. ERGs should be performed to know about retinal function (a, b and c waves: scotopic).

Reply 4: Thanks a lot for your constructive advice.

We examined the a-, b- and c-wave amplitudes of scotopic ERGs from WT and LCN2-TG mice to assess retinal function. Compared with WT mice, the amplitudes of both a-wave and c-wave had no significant difference, but the amplitude of b-waves was severely reduced after RIR injury in LCN2-TG mice. (Supplementary Figure 3A-3E). These findings demonstrated that upregulated LCN2 exacerbated retinal damage and plays more harmful effects in the RIR injury model.



Supplementary Figure 3. Electroretinography functional assessment.
(A-E) Electroretinography responses of WT and LCN2-TG mice in left eyes (without RIR injury) and in right eyes (with RIR injury) showing a-, b- and c-wave. Shown in (A, B) are representative individual responses of a-, b- and c-wave, and (C, D, E) are amplitudes of a-, b- and c-wave response peaks. n = 6 mice per group. All data represent mean ± SEM. *P < 0.05, **P < 0.01, ns: no significance. Statistical analysis was performed with unpaired two-tailed student's t-test.
Changes in the text: We added some data in our manuscript as advised (see results section, page 14 to 15, line 276 to 281, and see supplementary figure 3).

Re-review comments

Reviewer B

In the revised version of the manuscript, Mei et al have included additional results that support their claims. Importantly, they performed the LPX-1 treatment in LCN2-TG (Supplementary Fig. 6) and included the LCN2+/- (heterozygous line with a decreased level of LCN2; generated by CRISPR/CAS9) (Figure 6). These additional results are in line with the conclusions of the study and have improved the quality of the manuscript. I have a number of minor comments to improve

the manuscript; I leave it up to the authors and editors to decide whether to include them or not. Additionally, there are still a number of grammatical revisions necessary before the manuscript should be published. I have made extensive notes on this, but the manuscript should be proofread carefully to make sure the edits match the authors intentions and are grammatically and scientifically correct.

Suggestion 1:

The authors write: "These findings demonstrated that LCN2 induced ferroptosis could be inhibited by liproxstatin-1, and inhibiting retinal ferroptosis could efficiently restore RGC death and vision function impairment in RIR injury model." This seems an overstatement, as liproxstatin-1 could only partially restore RGC death and vision impairment. I suggest changing "efficiently" to "partially."

Reply 1: Thank you for your helpful comments and suggestions.

Changes in the text: We have corrected "efficiently" to "partially" in our manuscript (see results section, page 18, line 360).

Suggestion 2:

I encourage the authors to show the LCN2 protein level in LCN2-TG and LCN2+/- background at 1, 3, or 5 days after RIR injury as shown in Figure 1A and 1B for WT as an addition to main Figure 1 or supplementary data. It would be very interesting to see how LCN2 protein levels are effected in LCN2-TG and LCN2+/- background after RIR and compare that with WT.

Reply 2: Thank you for your helpful comments and suggestions.

Due to insufficient LCN2-TG and LCN2+/- mice currently, we will check the change of LCN2 protein level in LCN2-TG and LCN2+/- mice by western blot at 1, 3, or 5 days after RIR injury in our future study.

Grammar and clarity comments:

Comment 1: It was difficult to follow/locate the revised portion because there was a mismatch between the line numbers in the revised file and the line numbers mentioned in the comments to the reviewer. Hopefully there was not a mix up of some kind in the file submission process.

Reply 1: Thank you for your helpful suggestions.

I don't know why the line number in the modified file does not match the line number mentioned in the comments to the reviewer. The line number we modified this time is corresponding to the revised manuscript (ATM-22-3298-R2 (make all necessary revision to this version)-zhao).

Comment 2: The surname of the corresponding author (Zhao) should be capitalized on lines 16, 18, and 19.

Reply 2: Thank you for your helpful suggestions.

Changes in the text: We have corrected "the corresponding author (zhao)" to "Zhao" in our manuscript. (see page 1, line 16, 18, and 19).

Comment 3: Line 36, rephrase "performed" with "utilized" or words with a similar sense.

Reply 3: Thank you for your helpful suggestions.

Changes in the text: We have corrected "performed" to "utilized" in our manuscript (see abstract section, page 3, line 36).

Comment 4: Line 51, rephrase "can significantly ameliorate" with "could significantly ameliorate" or "significantly ameliorated" to match the consistent use of past tense in the paragraph.

Reply 4: Thank you for your helpful suggestions.

Changes in the text: We have corrected "can significantly ameliorate" to "could significantly ameliorate" in our manuscript (see abstract section, page 3, line 51).

Comment 5: Line 55, rephrase "upregulated LCN2 through promoting ferroptosis" with "upregulated LCN2 mediated promotion of ferroptosis"

Reply 5: Thank you for your helpful suggestions.

Changes in the text: We have corrected "upregulated LCN2 through promoting ferroptosis" to "upregulated LCN2 mediated promotion of ferroptosis" in our manuscript (see abstract section, page 4, line 56 and 57).

Comment 6: Line 73, remove extra space

Reply 6: Thank you for your helpful suggestions.

Changes in the text: We have removed extra space in our manuscript (see introduction section, page 5, line 72).

Comment 7: Line 98, rephrase “homozygosis” with “homozygous”

Reply 7: Thank you for your helpful suggestions.

Changes in the text: We have corrected “homozygosis” to “homozygous” in our manuscript (see methods section, page 6, line 102).

Comment 8: Line 96, rephrase the methods subsection “animals” with “experimental model”

Reply 8: Thank you for your helpful suggestions.

Changes in the text: We have corrected “animals” to “experimental model” in our manuscript (see methods section, page 6, line 100).

Comment 9: Lines 97 to 100, in the methods section “animals” describe the generation of LCN2 knock out homozygous mutant (LCN2-/-) and knock down heterozygous (LCN2+/-) by CRISPR/CAS9 system. Since (LCN2-/-) was not used in this study, only describe the heterozygous (LCN2+/-) line. State the guide RNA sequences used for the generation of CRISPR/CAS9 mutants.

Reply 9: Thank you for your helpful suggestions.

Changes in the text: We have corrected “homozygous” to “heterozygous” in our manuscript (see methods section, page 6, line 102).

Comment 10: Line 105, rephrase “, that is” with “in”, replace “.” with “,”, delete “:”

Reply 10: Thank you for your helpful suggestions.

Changes in the text: We have corrected “that is” to “in” and corrected “.” to “,” and deleted “:” in our manuscript (see methods section, page 7, line 107, 108, 109, 110, and 111).

Comment 11: Line 106, delete “were”

Reply 11: Thank you for your helpful suggestions.

Changes in the text: We have deleted “were” in our manuscript (see methods section, page 6, line

106).

Comment 12: Line 108, delete “were”

Reply 11: Thank you for your helpful suggestions.

Changes in the text: We have deleted “were” in our manuscript (see methods section, page 7, line 110).

Comment 14: Line 127, rephrase “coated with” with “applied on”

Reply 14: Thank you for your helpful suggestions.

Changes in the text: We have corrected “coated with” to “applied on” in our manuscript (see methods section, page 8, line 134).

Comment 15: Line 136, rephrase “freshly” with “fresh” or “freshly prepared”

Reply 15: Thank you for your helpful suggestions.

Changes in the text: We have corrected “freshly” to “fresh” in our manuscript (see methods section, page 8, line 144).

Comment 16: Line137, rephrase “has section of 5μm and” with ”(5 μm thick) was”

Reply 16: Thank you for your helpful suggestions.

Changes in the text: We have corrected “has section of 5μm and” to “(5 μm thick) was” in our manuscript (see methods section, page 8, line 145).

Comment 17: Line 142, rephrase “freshly” with “fresh” or “freshly prepared”

Reply 17: Thank you for your helpful suggestions.

Changes in the text: We have corrected “freshly” to “fresh” in our manuscript (see methods section, page 9, line 151).

Comment 18: Line 144, rephrase “thickness” with “thick sections”

Reply 18: Thank you for your helpful suggestions.

Changes in the text: We have corrected “thickness” to “thick sections” in our manuscript (see

methods section, page 9, line 153).

Comment 19: Line 146, delete “were”

Reply 19: Thank you for your helpful suggestions.

Changes in the text: We have deleted “were” in our manuscript (see methods section, page 9, line 156).

Comment 20: Line 163, delete “were”

Reply 20: Thank you for your helpful suggestions.

Changes in the text: We have deleted “were” in our manuscript (see methods section, page 9, line 165).

Comment 21: Line 171, rephrase “was showed in” with “shown as”

Reply 21: Thank you for your helpful suggestions.

Changes in the text: We have corrected “was showed in” to “shown as” in our manuscript (see methods section, page 10, line 181).

Comment 22: In the methods subsection “Electroretinopathy”, mention about a- and b- waves acquisitions also

Reply 22: Thank you for your helpful suggestions.

Changes in the text: We have added “a- and b- waves acquisitions” in our manuscript (see methods section, page 10, line 188 and 189).

Comment 23: Line 183, rephrase “was” with “were”

Reply 23: Thank you for your helpful suggestions.

Changes in the text: We have corrected “was” to “were” in our manuscript (see methods section, page 10, line 193).

Comment 24: Line 200, spell out the full form of RIPA, “Radio-Immunoprecipitation Assay”

Reply 24: Thank you for your helpful suggestions.

Changes in the text: We have corrected “RIPA” to “Radio-Immunoprecipitation Assay (RIPA)” in our manuscript (see methods section, page 11, line 210).

Comment 24: Method subsection “western blotting”, lines 200 to 202, rephrase as “RIPA lysis buffer (Beyotime Biotechnology, Shanghai, China) containing protease and phosphatase inhibitor (Beyotime Biotechnology, Shanghai, China) was added into dissected retinas”, to match the consistent use of passive voice.

Reply 24: Thank you for your helpful suggestions.

Changes in the text: We have corrected “into dissected retinas” to “was added into dissected retinas” in our manuscript (see methods section, page 11, line 212).

Comment 25: Method subsection “western blotting”, mentions about the positive control “tubulin” and normalization method. A sentence, “The protein levels were normalized to tubulin and expressed relative to WT or control” or other sentences with similar sense could be added in the western blotting method section. Include the information in the figure legends of all main and supplementary figures, containing western blot and protein level quantification, with context-specific modifications.

Reply 25: Thank you for your helpful suggestions.

Changes in the text: We have added “The protein levels were normalized to tubulin and expressed relative to WT or control” in our manuscript (see methods section, page 12, line 224 and 225).

Comment 26: Lines 219 and 220, there is a mismatch between the full form and abbreviated form of RT-qPCR

Reply 26: Thank you for your helpful suggestions.

Changes in the text: We have corrected “Quantitative real-time PCR (RT-qPCR)” to “Quantitative PCR (qPCR)” in our manuscript (see methods section, page 12, line 231 and 232).

Comment 27: Lines 221 and 222, rephrase the sentence as “mRNA expression levels were normalized to GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE (GADPH) control and expressed relative to WT”. Make sure to italicize gene or RNA names. Mention the sentence in

Figure 4 legend also.

Reply 27: Thank you for your helpful suggestions.

Changes in the text: We have corrected “measured and normalized results to GAPDH control” to “normalized to GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE (*GADPH*) control and expressed relative to WT” in our manuscript (see methods section, page 12, line 234 and 235).

Comment 28: Line 225, rephrase “Data were showed” with “Data shown”

Reply 28: Thank you for your helpful suggestions.

Changes in the text: We have corrected “Data were showed” to “Data shown” in our manuscript (see methods section, page 13, line 239).

Comment 29: Line 236, rephrase “treatment” with “RIR injury”

Reply 29: Thank you for your helpful suggestions.

Changes in the text: We have corrected “treatment” to “RIR injury” in our manuscript (see results section, page 13, line 251).

Comment 30: Line 237, rephrase “LCN2 is involved” with “LCN2 might be involved” since it is the starting/ first data where a correlation between elevated LCN2 and RIR injury was observed.

Reply 30: Thank you for your helpful suggestions.

Changes in the text: We have corrected “LCN2 is involved” to “LCN2 might be involved” in our manuscript (see results section, page 13, line 252).

Comment 31: Lines 239 and 240, capitalize and italicize the gene name as “LCN2”, here and throughout the manuscript

Reply 31: Thank you for your helpful suggestions.

Changes in the text: We have corrected “LCN2” to “*LCN2*” in our manuscript (see results section, page 13, line 255).

Comment 32: Line 241, italicize as “LCN2-TG” mice as it is genetic background of mice, here and throughout the manuscript

Reply 32: Thank you for your helpful suggestions.

Changes in the text: We have corrected "LCN2-TG" to "*LCN2-TG*" in our manuscript (see results section, page 13, line 257).

Comment 33: Line 246, delete the redundant word "expressed"

Reply 33: Thank you for your helpful suggestions.

Changes in the text: We have deleted the redundant word "expressed" in our manuscript (see results section, page 14, line 262).

Comment 34: Line 260, rephrase "with" with "by" and "by" with "of"

Reply 34: Thank you for your helpful suggestions.

Changes in the text: We have corrected "with" to "by" and "by" to "of" in our manuscript (see results section, page 14, line 276).

Comment 35: Line 268, rephrase as "…mice (control group),"

Reply 35: Thank you for your helpful suggestions.

Changes in the text: We have corrected "both WT and LCN2-TG mice" to "both WT and LCN2-TG mice (control group)" in our manuscript (see results section, page 15, line 285).

Comment 36: Line 276, rephrase "plays" with "exerted"

Reply 36: Thank you for your helpful suggestions.

Changes in the text: We have corrected "plays" to "exerted" in our manuscript (see results section, page 15, line 292).

Comment 37: Line 291, rephrase "level were showed" with "level was seen"

Reply 37: Thank you for your helpful suggestions.

Changes in the text: We have corrected "level were showed" to "level was seen" in our manuscript (see results section, page 16, line 307).

Comment 38: Line 293, Capitalize W in "we"

Reply 38: Thank you for your helpful suggestions.

Changes in the text: We have corrected “we” to “We” in our manuscript (see results section, page 16, line 310).

Comment 39: Line 294, delete “expression” for consistency

Reply 39: Thank you for your helpful suggestions.

Changes in the text: We have deleted “expression” in our manuscript (see results section, page 16, line 310).

Comment 40: Line 296 and 297, rephrase “, and it is divided” into “composed of”

Reply 40: Thank you for your helpful suggestions.

Changes in the text: We have corrected “, and it is divided” to “composed of” in our manuscript (see results section, page 16, line 313).

Comment 41: Between lines 297 and 298, add a sentence telling what is already known about the correlation between the level of Ferritin chains and ferroptosis.

Reply 41: Thank you for your helpful suggestions.

Changes in the text: We have added “Ferritin plays a key role in protecting against ferroptosis” in our manuscript (see results section, page 16, line 314).

Comment 42: Line 304, delete extra space between “, and we”

Reply 42: Thank you for your helpful suggestions.

Changes in the text: We have deleted extra space between “, and we” in our manuscript (see results section, page 16, line 322).

Comment 43: Line 307, rephrase “immunology” with “immuno”

Reply 43: Thank you for your helpful suggestions.

Changes in the text: We have corrected “immunology” to “immune” in our manuscript (see results section, page 16, line 326).

Comment 44: Line 312, delete “expression of”

Reply 44: Thank you for your helpful suggestions.

Changes in the text: We have deleted “expression of” in our manuscript (see results section, page 17, line 332).

Comment 45: Line 316, rephrase “whether induced” with “whether induced elevation of”

Reply 45: Thank you for your helpful suggestions.

Changes in the text: We have corrected “whether induced” to “whether induced elevation of” in our manuscript (see results section, page 17, line 334).

Comment 46: Line 321, rephrase “as well as the expression” with “ as well as induced the expression”

Reply 46: Thank you for your helpful suggestions.

Changes in the text: We have corrected “as well as the expression” to “as well as induced the expression” in our manuscript (see results section, page 17, line 340).

Comment 47: Line 333, rephrase “injured” with “injury”

Reply 47: Thank you for your helpful suggestions.

Changes in the text: We have corrected “injured” to “injury” in our manuscript (see results section, page 18, line 352).

Comment 48: Line 341, rephrase “could efficiently restore” with “could alleviate”.

“could efficiently restore RGC cell death and visual impairment” can mean that the rescue is 100% or like WT/control, clearly not the case here

Reply 48: Thank you for your helpful suggestions.

Changes in the text: We have corrected “could efficiently restore” to “could partially restore” in our manuscript (see results section, page 18, line 360).

Comment 49: Line 346, rephrase “performed” with “used”

Reply 49: Thank you for your helpful suggestions.

Changes in the text: We have corrected “performed” to “used” in our manuscript (see results

section, page 18, line 365).

Comment 50: Line 346, rephrase “LCN2 knockout” with “LCN2+/- knockdown” as only LCN2+/- has been used.

Reply 50: Thank you for your helpful suggestions.

Changes in the text: We have corrected “LCN2 knockout” to “$LCN2^{+/-}$ knockdown” in our manuscript (see results section, page 18, line 365).

Comment 51: Line 348, rephrase “Lcn2” with “LCN2”

Reply 51: Thank you for your helpful suggestions.

Changes in the text: We have corrected “Lcn2” to “LCN2” in our manuscript (see results section, page 18, line 367).

Comment 53: Line 369, delete “different time points”

Reply 53: Thank you for your helpful suggestions.

Changes in the text: We have deleted “different time points” in our manuscript (see results section, page 19, line 389).

Comment 54: In the discussion section, please include references to the main and supplementary figures whenever the results from this study are mentioned, for example, however, compared with WT mice, the retinas of LCN2 TG mice exhibit more grievous damage with RIR treatment (Figure XX, Supplementary Figure XX, etc., …

Reply 54: Thank you for your helpful suggestions.

Changes in the text: We have added references to the main and supplementary figures in our manuscript (see discussion section, page 19, line 389, page 20, line 410, page 21, line 423, 425, and 426).

Comment 55: Line 378, rephrase “treatment” with “injury”

Reply 55: Thank you for your helpful suggestions.

Changes in the text: We have corrected “treatment” to “injury” in our manuscript (see results

section, page 20, line 398).

Comment 56: Line 380, rephrase “might be act” with “might be acting” or “might act”

Reply 56: Thank you for your helpful suggestions.

Changes in the text: We have corrected “might be act” to “might be acting” in our manuscript (see results section, page 20, line 400).

Comment 57: Line 382, rephrase “might be served” as “might serve”

Reply 57: Thank you for your helpful suggestions.

Changes in the text: We have corrected “might be served” to “might serve” in our manuscript (see results section, page 20, line 402).

Comment 58: Line 383, rephrase “and it worked” with “working”

Reply 58: Thank you for your helpful suggestions.

Changes in the text: We have corrected “and it worked” to “working” in our manuscript (see results section, page 20, line 403).

Comment 59: Line 400, rephrase as “shown as significantly” with “as evident from significantly”

Reply 59: Thank you for your helpful suggestions.

Changes in the text: We have corrected “shown as significantly” to “as evident from significantly” in our manuscript (see results section, page 21, line 421).

Comment 60: Line 411, rephrase “a critical” with “an important” since there are no experiments on knockout mice and in knockdown heterozygous mice with about 50% reduction in protein level, only about 10 to 15% rescue of RGC death was seen suggesting an involvement of some other important regulators as well.

Reply 60: Thank you for your helpful suggestions.

Changes in the text: We have corrected “a critical” to “an important” in our manuscript (see results section, page 21, line 433).

Comment 61: Line 412, rephrase “lead” with “leads”

Reply 61: Thank you for your helpful suggestions.

Changes in the text: We have corrected “lead” to “leads” in our manuscript (see results section, page 21, line 434).

Comment 62: Line 414, rephrase “will”, a word that denotes the certainty with “may”

Reply 62: Thank you for your helpful suggestions.

Changes in the text: We have corrected “will” to “may” in our manuscript (see results section, page 21, line 437).

Comment 63: Lines 417 to 426 abbreviations section, if authors decide to include abbreviations and full forms of some genes/proteins like LCN2, GPX4, etc., here, then they should include all of the genes included in this study, like GAPPH, IL6, IL1β, CCl2, CCL3, etc. Spell out the full form of SEM (Standard error of mean)

Reply 63: Thank you for your helpful suggestions.

Changes in the text: We have added full forms of some genes/proteins like GAPPH, IL6, IL1β, CCl2, CCL3 in our manuscript (see abbreviations section, page 23, line 443, 444, and 445).

Comment 63: Line 562, in Figure 1E, demarcate the layers as in Figure 2A. Spell out the full form of I/R in legend. For consistency, I recommend using RIR instead of I/R throughout the text, figures, and legends.

Reply 63: Thank you for your helpful suggestions.

Changes in the text: We have corrected “RIR” to “retinal ischemia/reperfusion” in legend and demarcated the layers as in Figure 1E (see figure legends section, page 29, line 599, 600 and 612, page 30, line 633, page 31, line 662, page 32, line 680).

Comment 64: Line 578, rephrase “in” with “from” here and other places, lines 601, 617, 631, and 650

Reply 64: Thank you for your helpful suggestions.

Changes in the text: We have corrected “in” to “from” in our manuscript (see figure legends section,

page 29, line 613, 635, 650, 663, and 681).

Comment 65: Figure 3, add scale bar information for Figure 3E.

Reply 65: Thank you for your helpful suggestions.

Changes in the text: We have added scale bar information for Figure 3E in our manuscript (see figure legends section, page 31, line 645).

Comment 66: Figure 4, italicize gene names in Figure 4C, 4D, 4E, and 4F.

Reply 66: Thank you for your helpful suggestions.

Changes in the text: We have italicized gene names (*IL1β, IL6, CCL2* and *CCL3*) in Figure 3C, 3D, 3E, and 3F in our manuscript (see figure legends section, page 31, line 655).

Comment 67: Figure 4, in Figure 4B, unlike activated Muller glia cells (Figure 4A), it looks like there is a difference not only in fluorescence intensity in double immunostaining (Supplementary Figure but also in the number of activated microglia cell numbers. Hence, a graph showing the quantification of activated microglia as a main or supplementary figure would be very useful.

Reply 67: Thank you for your helpful suggestions.

Changes in the text: We have added a graph showing the quantification of activated microglia as a supplementary figure in our manuscript (see supplementary figure section, supplementary figure 5).

Comment 68: Line 618, rephrase “Double immunology staining” with “double immune staining”, here and throughout the text and figure legends, lines, 702, 709

Reply 68: Thank you for your helpful suggestions.

Changes in the text: We have corrected “Double immunology staining” to “double immune staining” in our manuscript (see figure legends section, page 31, line 651).

Comment 69: Line 620, rephrase “showed that” with “showing”, here, and lines 622, 655, and 719

Reply 69: Thank you for your helpful suggestions.

Changes in the text: We have corrected “showed that” to “showing” in our manuscript (see figure

legends section, page 31, line 653, page 32, line 664, page 33, line 686).

Comment 70: Line 669 to 671, supplementary figure 1 and legend, all LCN2 should be capitalized and italicized (LCN2) in the figure as well as legend

Reply 70: Thank you for your helpful suggestions.

Changes in the text: We have corrected "LCN2" to "*LCN2*" in supplementary figure 1 and legend (see supplementary figure legends section, supplementary figure 1).

Comment 71: Line 683, supplementary figure 2B, since HA-tagged LCN2 is not expressed in WT, the expression LCN2 level in LCN2-TG background relative to WT does not make much sense. Hence the HA-tagged LCN2 level in WT could be shown as zero and that in the LCN2-TG background could be shown as an absolute numerical value.

Reply 71: Thank you for your helpful suggestions.

Changes in the text: We have modified the figure according to your requirements in our manuscript (see supplementary figure legends section, supplementary figure 2).

Comment 72: Line 714, delete the redundant line "All data represent mean +/- SEM"

Reply 72: Thank you for your helpful suggestions.

Changes in the text: We have deleted the redundant line "All data represent mean ± SEM" in our manuscript (see supplementary figure legends section, supplementary figure 2).

Comment 73: Line 702 and 711, supplementary figures 4 and 5 have one graph each, and hence no need to mention (A) and hence delete them. Also in the text refer as supplementary figure 4 and supplementary figure 5, not supplementary figure 4A (line 311) and supplementary figure 5A (line 315)

Reply 73: Thank you for your helpful suggestions.

Changes in the text: We have corrected "supplementary figures 4A and 5A" to "supplementary figures 4 and 5" in our manuscript (see supplementary figure legends section, supplementary figure 4 and 5).

Comment 74: Line 732, rephrase “response” with “responses”

Reply 74: Thank you for your helpful suggestions.

Changes in the text: We have corrected “response” to “responses” in our manuscript (see supplementary figure legends section, supplementary figure 6).

Comment 75: Line 744, italicize the gene names

Reply 75: Thank you for your helpful suggestions.

Changes in the text: We have italicized the gene names in our manuscript.